

**MICROBES AND MONITORING TOOLS FOR ANAEROBIC CHLORINATED
METHANE BIOREMEDIATION**

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By

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**MICROBES AND MONITORING TOOLS FOR ANAEROBIC CHLORINATED
METHANE BIOREMEDIATION**

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You have brains in your head.
You have feet in your shoes.
You can steer yourself in any direction you choose.
You're on your own.
And you know what you know.
You are the guy who'll decide where to go.

Dr. Seuss

This is for you, Angel, Mom and Dad.
Thanks for always being there for me,
and for believing that I can accomplish great things.

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LIST OF SYMBOLS OR ABBREVIATIONS

1,1,1-TCA	1,1,1-trichloroethane
¹² C	light stable isotope of carbon
¹³ C	heavy stable isotope of carbon
2-BES	2-bromoethanesulfonate
2,5-DCB	2,5-dichlorobenzoate
3-CBA	3-chlorobenzoate
ABI	Applied Biosystems
ASM	American Society for Microbiology
ATSDR	Agency for Toxic Substances and Disease Registry
BLAST	Basic Local Alignment Search Tool
bp	base pair
CF	chloroform
Cl ⁻	chloride ion
CM	chloromethane
CSIA	compound-specific isotope analysis
CT	carbon tetrachloride
DCE	<i>cis</i> -dichloroethene
DCM	dichloromethane
DGGE	denaturing gradient gel electrophoresis
DIRB	dissimilatory ferric iron-reducing bacteria
DNA	deoxyribonucleic acid
ECD	electron capture detector
EPA	U.S. Environmental Protection Agency
g	gravitational constant
GAANN	Graduate Assistance in Areas of National Need
GC	gas chromatography
GC-IRMS	gas chromatography-isotope ratio mass spectrometry
GC/C/IRMS	gas chromatography/combustion/isotope ratio mass spectrometry
HCFC-22	monochlorodifluoromethane
HCl	hydrochloric acid
HGT	horizontal gene transfer
HP	Hewlett-Packard
IRMS	isotope ratio mass spectrometry
ISCO	<i>in situ</i> chemical oxidation
KIE	kinetic isotope effect
KIEs	kinetic isotope effects
kJ	kilojoules
L	liter
MBTs	molecular biology tools
MCL	maximum contaminant level
mg	milligrams
MGB	minor groove binding
min	minute(s)

mM	millimolar
NaHCO ₃	sodium bicarbonate
NPL	national priority list
OTU	operational taxonomic unit
PBDE	polybrominated diphenyl ethers
PCE	tetrachloroethene
PCR	polymerase chain reaction
PD	phylogenetic distance
PRB	permeable reactive barrier
psig	
qPCR	quantitative real-time PCR
RDase	reductive dehalogenase
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
SEAR	surfactant enhanced aquifer remediation
SERDP	Strategic Environmental Research and Development Program
sp.	species (singular)
SPL	substance priority list
spp.	Species (plural)
TCE	trichloroethene
vol/vol	volume per volume
ϵ_{bulk}	bulk stable carbon isotopes enrichment factor
μL	microliter
μM	micromolar

SUMMARY

The chlorinated methanes carbon tetrachloride (CT), chloroform (CF), dichloromethane (DCM) and chloromethane (CM) are widespread groundwater pollutants that pose risks to human and ecosystem health. Although some progress has been made in elucidating the microbiology contributing to the aerobic degradation of DCM and CM, these efforts have had little impact on bioremediation practices aimed at restoring anoxic aquifers impacted by chlorinated methanes. Remaining knowledge gaps include the lack of understanding of the microbial mechanisms and pathways contributing to chlorinated methane transformations under anoxic conditions. Thus, the major goals of this research effort were to identify microbes that can contribute to the transformation of chlorinated methanes in the absence of oxygen, and to develop monitoring tools to assess anaerobic chlorinated methane bioremediation at contaminated sites. To accomplish these goals, freshwater and estuarine sediment samples from 45 geographically distinct locations, including 3 sites with reported chlorinated-methane contamination, were collected and screened for CT-, CF-, DCM- and/or CM-degrading activity. DCM degradation was observed in microcosms established with sediment materials from 15 locations, and the sediment-free, DCM-degrading enrichment culture RM was obtained from Rio Mameyes sediment. 16S rRNA-gene based community analysis characterized consortium RM, and identified a *Dehalobacter* sp. involved in DCM fermentation to non-toxic products. Organism- and process-specific monitoring tools were designed that target the 16S rRNA gene of the DCM-fermenting *Dehalobacter* sp. and the consortium's specific ^{13}C -DCM enrichment factor, respectively. Treatability studies using site materials that showed no chlorinated methane degradation activity demonstrated the feasibility of using CF- and DCM-degrading consortia for

bioaugmentation applications. Collectively, this study expands our understanding of bacteria contributing to chlorinated methane degradation, provides new tools for monitoring anaerobic DCM degradation, and demonstrates that microbial remedies at chlorinated methane contaminated sites are feasible.

CHAPTER 1

INTRODUCTION

1.1 Thesis Rationale

Carbon tetrachloride (CT), chloroform (CF), dichloromethane (DCM) and chloromethane (CM) are widespread groundwater pollutants that pose risks to human and ecosystem health. Although some progress has been made in elucidating the mechanisms and pathways contributing to the degradation of chlorinated methanes, these efforts have had little impact on bioremediation practices aimed at restoring sites impacted by CT, CF, DCM and CM. Remaining knowledge gaps include the lack of understanding of the microbiology contributing to chlorinated methane transformations and the applicability of bioremediation approaches at sites impacted with chlorinated methanes. In addition, knowledge of the interplay of microbially-mediated reactions with abiotic transformation processes remains rudimentary to date. With the availability of new tools to characterize active community members, novel expertise for enriching and isolating microorganisms involved in chlorinated solvent degradation and focus on *in situ* bioremediation strategies and coupled biotic-abiotic processes, significant opportunities exist to advance microbial remedies to tackle chlorinated methane contamination.

1.2 Goals and Research Objectives

The current lack of knowledge of microorganisms and the dominant transformation mechanisms that contribute to the fate of chlorinated methanes limits our ability to

predict and control the environmental fate of these contaminants. The major goal of this research effort is identify microbes that contribute to the anaerobic transformation of chlorinated methanes and to develop monitoring tools to assess the anaerobic biodegradation of chlorinated methane at contaminated sites.

1.2.1 Objective 1: Explore sediment and aquifer materials collected from contaminated and pristine sites for the presence of microorganisms that metabolize chlorinated methanes.

1.2.2 Objective 2: Establish microcosms and enrichment cultures that transform chlorinated methanes.

1.2.3 Objective 3: Identify bacteria involved in the metabolism of chlorinated methanes using molecular biological tools (MBTs) and high-throughput sequencing technologies.

1.2.4 Objective 4: Develop compound-specific isotope analysis (CSIA) as a tool to monitor degradation, estimate reaction rates and elucidate transformation mechanisms for chlorinated methanes.

1.3 Literature Review

1.3.1 Background

Chlorinated methanes – carbon tetrachloride (CT), chloroform (CF), dichloromethane (DCM), and chloromethane (CM) – have been produced at a scale of several million tons per year by the chemical industry (25). Improper handling and storage have led to the release of significant amounts of these chemicals into the environment, and the U.S.

Environmental Protection Agency (EPA) has classified chlorinated methanes as priority contaminants with known or suspected carcinogenic or mutagenic effects (67, 70).

Halogenated methanes are also emitted in substantial amounts from natural sources such as marine algae, fungi, phytoplankton, brush and forest fires, and volcanoes (46, 48, 49, 65, 79, 81, 87, 125). The natural production of halogenated methanes suggests that microorganisms have been exposed to these compounds for billions of years, and thus had ample time to evolve mechanisms to degrade and benefit from halogenated methanes as growth substrates. The recognition of extensive natural production of halogenated methanes without apparent accumulation in the environment indicates that natural transformation and degradation mechanisms exist. The overarching goal of this research effort is to elucidate the microbiology and the mechanisms contributing to the natural cycling of halogenated methanes and to use this information to understand and control the transformation and fate of chlorinated methanes at contaminated sites. Similar to what has been accomplished for chlorinated ethenes, this research effort aims to identify groups of microorganisms that efficiently transform and detoxify chlorinated methanes to reduce near- and long-term environmental and health risks.

1.3.2 Production, use and release of chlorinated methanes

CT is produced by chlorination of a variety of low molecular weight hydrocarbons such as CM, carbon disulfide, methane, ethane, propane, and ethylene dichloride (Figure 1.1) (101). Serving as feedstock for the chemical synthesis of fluorochlorocarbons, and formerly used as dry cleaning agent, solvent, catalyst, in fire extinguishers and in the chemical manufacture of polymers, the production of CT in the U.S. peaked in 1974 (101). Since then, the production of CT has declined at approximately 8% per year as a

result of the adoption of the Montreal Protocol – an international agreement to reduce environmental concentrations of ozone-depleting chemicals (including CT) – and because of the provisions of Title VI of the Clean Air Act Amendments of 1990 addressing these chemicals (30, 107). The release of CT into soil and subsurface waters occurs due to spills, run-off from agricultural soils, dumping and through landfill leaching, mostly resulting from industrial and agricultural practices (1). Once released into the environment, CT has the potential to cause liver, kidney and lung damage and liver cancer upon human exposure to concentrations above the maximum contaminant level (MCL) in drinking water, set by the U.S. EPA to $0.005 \text{ mg CT L}^{-1}$ (1, 33). CT is also produced via natural processes. Natural production of CT has been reported in terrestrial plants, during the decomposition of organic matter in tropical forest soils, and in the gases emitted by volcanoes (47, 68, 80).

Commercial production of CF occurs mainly via two methods: chlorination of methane or CM produced by the reaction of methanol and hydrochloric acid (HCl) (Figure 1.1) (2). In the past, CF was used as an anesthetic and in dry cleaning products, in fire extinguishers, and as a fumigant (2, 39). Most of the CF currently produced in the U.S. is used in the chemical manufacture of monochlorodifluoromethane (HCFC-22), a commonly used refrigerant scheduled for 99.5% phase-out in 2020 because of its ozone depletion and global warming potential (37, 102). CF may be released to the air, soil, and groundwater from a large number of sources related to its production and use, but human exposure to CF mainly occurs through contaminated drinking water. Previous reports suggest that exposure to CF may result in developmental abnormalities, damage to liver and central nervous system, and gastrointestinal cancers (2, 34). Because of the possible detrimental effects associated with CF exposure, the U.S. EPA has set a MCL

of total trihalomethanes in drinking water – including bromodichloromethane, bromoform, dibromochloromethane and CF – of 0.080 mg L^{-1} (36). Interestingly, estimated emissions of CF from anthropogenic sources only account for roughly 10% of the estimated total emissions from all sources (80). Natural production of CF has been observed in fungi, lichens and several species of macro and microalgae, during the decomposition of organic matter, and within termite mounds (47, 58, 59, 81, 105). In particular, the flux of CF from termite mounds is thought to account for as much as 15% of global CF emissions (47, 81).

The chlorination of methane and CM is also used to manufacture DCM for industrial applications (Figure 1.1) (4, 103). DCM is predominantly used as a solvent in the pharmaceutical, electronics manufacturing and paint and coating industries (39). Additionally, DCM has been used for agricultural purposes as a fumigant and degreening (i.e., ripening) agent (103). Although natural production of DCM has been observed in several temperate macroalgal species, one microalgae and a few phytoplankton phylotypes, the majority of DCM emissions are from anthropogenic sources (4, 47, 112). Thus, DCM is released into the air, soil and subsurface environments mainly due to improper handling and disposal practices during industrial and consumer uses. DCM is contained in many chemical waste sites, including National Priority List (NPL) sites across the U.S. and its territories (4). Human exposure to DCM as a result of breathing vapors of contaminated air or drinking water from contaminated wells can lead to damages to the liver, kidneys, central nervous system and cardiovascular system. Furthermore, the U.S. EPA considers DCM to be a probable human carcinogen with a MCL of 0.005 mg L^{-1} in drinking water (36).

CM is derived from the chlorination of methane via the reaction of HCl and methanol (Figure 1.1) (104). In the past, CM was widely used as a refrigerant, foam-blowing agent and pesticide (3). At present, most of the commercially produced CM is used to make silicones, agricultural chemicals, methylcellulose, quaternary amines and butyl rubber (3). Although low concentrations of CM are sometimes detected in municipal and industrial waste streams, many of the industrial processes that utilize CM make complete use of the CM feedstock, releasing little or no CM into the environment (3). Most of the CM released into the environment (estimated at up to 99%) comes from natural sources (3). Macro and microalgae, diatoms and other phytoplankton, and higher plant species such as potato cultivars are known natural CM producers (47). In addition, abundant CM production has been reported for coastal salt marshes, freshwater peatland sites and rice cultivation fields, and from biomass burning in grasslands and forested areas (47). Chronic exposure to CM can negatively affect heart, liver, kidney and nervous system function; however, the U.S. EPA does not consider CM to be a probable human carcinogen and no MCL in drinking water has been established to date (35, 39).

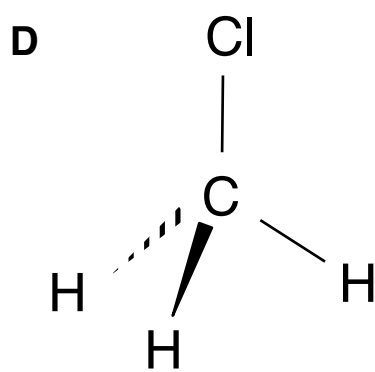
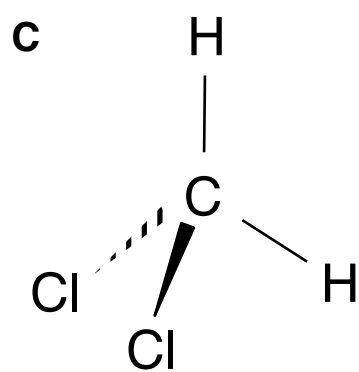
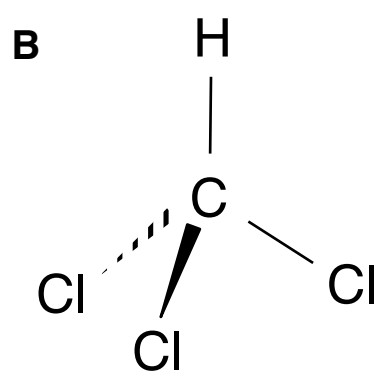
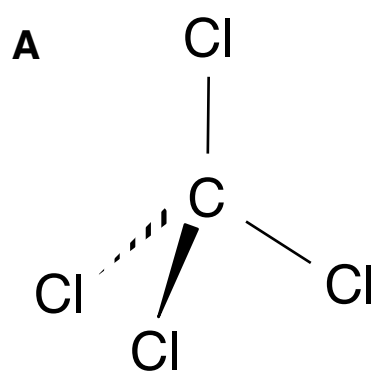


Figure 1.1 Chemical structures of chlorinated methanes: carbon tetrachloride (A), chloroform (B), dichloromethane (C) and chloromethane (D).

1.3.3 Transformation of chlorinated methanes

Transformation of CT, CF, DCM and CM has been reported in a number of laboratory studies and has been observed in some contaminated sediments and aquifers (19, 20, 114). The transformation of chlorinated methanes in these environments has been attributed to both biotic reactions involving the active metabolism of microorganisms and to abiotic reactions (41, 66); however, the microbiology and coupled abiotic mechanisms most relevant for achieving detoxification of chlorinated methanes at contaminated sites remain elusive.

1.3.3.1 Coupled microbial-abiotic transformation of chlorinated methanes

CT and CF have been shown to degrade in the presence of sulfide and sheet silicates, pyrite, iron(II)-coated goethite, chloride green rust, and ferrous sulfide (8, 13, 15, 16, 42, 52, 73, 74, 85, 93, 98). Several other reactive chemical reductants mediate the dechlorination of CT, CF and DCM. The abiotic reactions catalyzed by these reactive chemical species typically depend on microbial activity, which generates the reactive species/surfaces. Apparently, CT and CF transformation in an environmental setting can occur through a combination of both abiotic and biological processes, and the abiotic transformation of CT and lesser chlorinated methanes is indirectly controlled by microbial activity. The interplay between microbial formation of reactive chemical species and the coupled biotic-abiotic transformation of chlorinated methanes is poorly understood.

Reactive forms of iron(II) play key roles in abiotic transformation of chlorinated methanes and iron-bearing minerals are commonly present in aquifers. Dissimilatory ferric iron-reducing bacteria (DIRB) can generate reactive forms of iron(II) and contribute to the

abiotic transformation of CT in iron-bearing sediments. For example, in experiments performed by Amonette et al., biogenic iron(II) formed by the enzymatic reduction of goethite (a ferric iron oxyhydroxide) by the iron(III)-reducing bacterium *Shewanella alga* strain BrY, dechlorinated CT to CF as major end product (8). Similarly, McCormick et al. demonstrated that *Geobacter metallireducens* transformed CT through the formation of a reactive mineral surface comprised of nanoscale magnetite particles produced during ferric iron respiration (98). Since DIRB are ubiquitous in subsurface environments, biogenic minerals, such as those produced by *S. alga* and *G. metallireducens*, could play important roles for chlorinated methane transformation processes (88).

1.3.3.2 Cometabolic biotransformation of chlorinated methanes

Cometabolic transformation has no apparent benefit for the organism catalyzing the reaction(s) (5, 122). Since the transformation reactions are fortuitous (i.e., catalyzed by promiscuous enzyme systems), the bacteria require a carbon and energy source to support growth and sustain the cometabolic reaction(s). Transition metal cofactors of several bacterial enzyme systems are thought to be responsible for the cometabolic reductive dechlorination of chlorinated methanes (45, 75-77, 84). Although cometabolic reactions can lead to the detoxification of chlorinated methane-contaminated sediments or groundwater, cometabolic dechlorination is generally a slow process that cannot be easily manipulated (6, 86). Cometabolic conversions of target compounds depend on the microbial metabolism of a primary substrate and thus, are difficult to implement at the field scale (10).

1.3.3.3 Catabolic biotransformation of chlorinated methanes by specialized bacteria

1.3.3.3.1 Chlorinated methanes as a source of carbon and energy

Many bacteria grow while using chlorinated compounds with carbon-carbon bonds as the sole source of carbon and energy. To date, no bacteria that grow with CT or CF as a source of carbon have been described but growth with DCM and CM has been observed under oxic and anoxic conditions. Aerobic degradation of DCM and CM is mediated by glutathione-dependent dehalogenases that catalyze the conversion of DCM and CM into formaldehyde and inorganic chloride (Figure 1.2) (12, 53, 71, 72, 78, 99, 113). For both DCM and CM, glutathione initiates a nucleophilic substitution that displaces one halogen atom. Although aerobic degradation of DCM and CM is fairly well understood, these contaminants often reside in anoxic environments where aerobic metabolism plays no role. Anaerobic catabolism of DCM has been observed with homoacetogenic cultures DM and DC (11, 91). The acetogenic fermentation of DCM in culture DM was supported via interspecies formate transfer from strain DMA, a strictly-anaerobic, gram-positive, endospore-forming rod, to strain DMB, a strictly-anaerobic, gram-negative, endospore-forming homoacetogen (11). Culture DC contained a sulfate-reducing bacterium belonging to the *Desulfovibrio* genus and bacterium DMB, which was responsible for DCM degradation and acetate formation. Bacterium DMB was isolated and was the first pure culture with the ability to grow fermentatively with DCM. Via fermentation, bacterium DMB was able to derive energy from the oxidation of DCM to acetate in the absence of an exogenous electron acceptor (Figure 1.3). This organism was characterized as *Dehalobacterium formicoaceticum* (90, 92). Anaerobic catabolism of CM has also been reported. A methylotrophic acetogen initially named strain MC, now *Acetobacterium dehalogenans*, was isolated from sewage sludge with CM as energy source (121). Strain MC produces acetate as the main fermentation product.

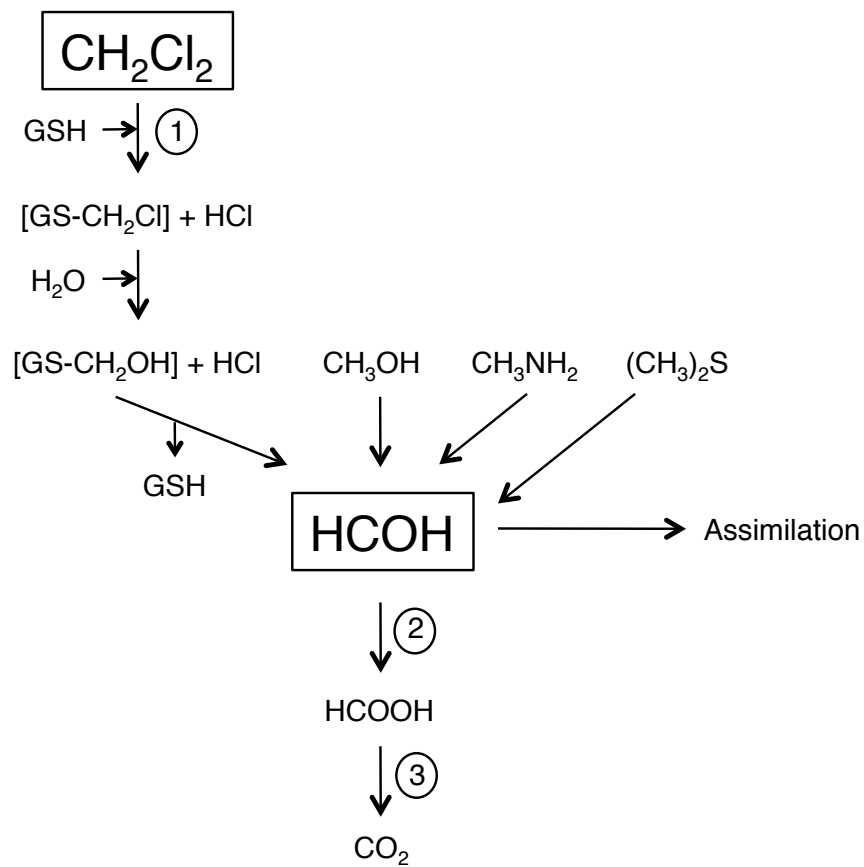


Figure 1.2 Aerobic metabolism of DCM by methylotrophic bacteria. 1, DCM dehalogenase; 2, formaldehyde dehydrogenase; 3, formate dehydrogenase. GSH stands for reduced glutathione. Image adapted from Leisinger et al. (82).

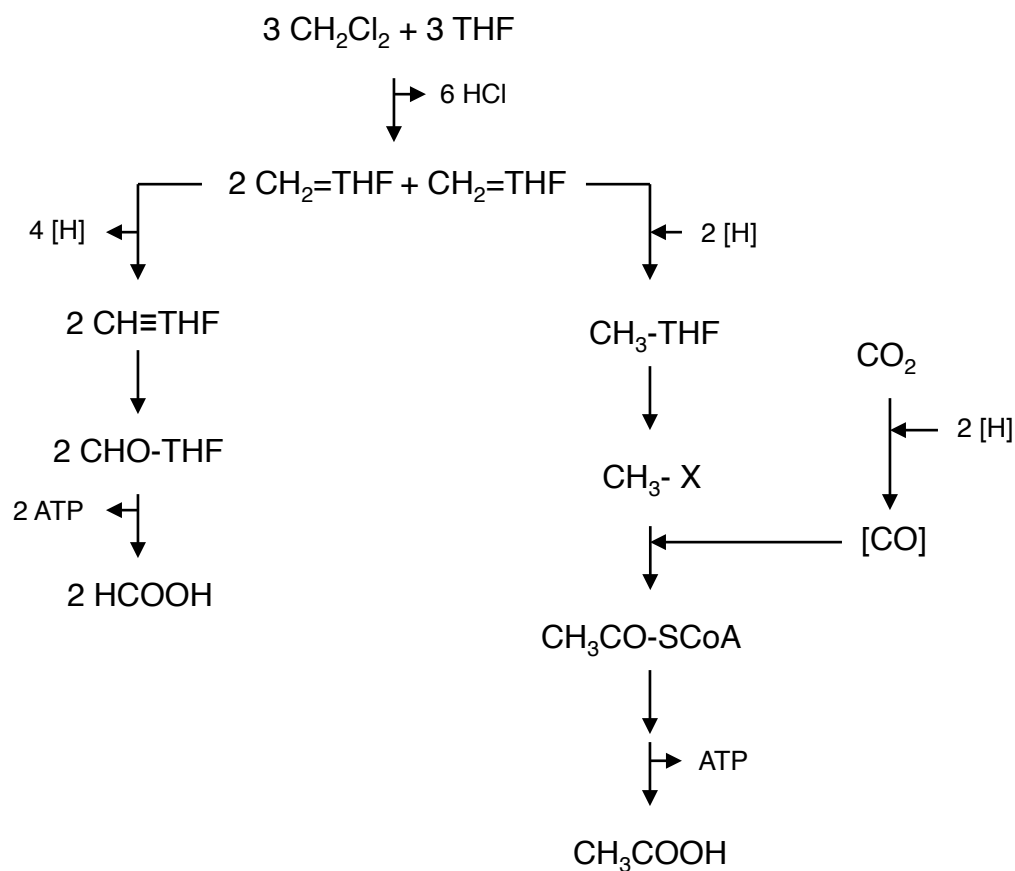


Figure 1.3 Proposed pathway for the fermentation of DCM by *Dehalobacterium formicoaceticum*. In this scheme, dichloromethane and tetrahydrofolate (THF) are converted by one or more unknown enzymatic reactions to methylene tetrahydrofolate and inorganic chloride. Two-thirds of the methylene tetrahydrofolate formed is then oxidized to formate by the enzymes of the acetyl coenzyme A (acetyl-CoA) pathway. The reducing equivalents generated by this oxidation are used by methylene tetrahydrofolate reductase and CO dehydrogenase in the formation of acetate from methylene tetrahydrofolate and CO_2 . X stands for a methyl carrier such as a corrinoid/iron-sulfur protein. Image adapted from Mägli et al. and Fantroussi (41, 90).

1.3.3.3.2 Chlorinated methanes as respiratory electron acceptors (organohalide respiration)

Another way of coupling growth to the transformation of chlorinated compounds is by using them as terminal electron acceptors for energy conservation, a process known as organohalide respiration (also called [de]halorespiration or respiratory reductive dehalogenation). Although a variety of bacteria have been isolated that grow with chlorinated ethenes and ethanes as electron acceptors (10, 54, 61, 96), only one bacterium has been described that respire a chlorinated methane (50). Since the energetics of chlorinated methane reductive dechlorination are favorable (26), it is reasonable to predict that a greater diversity of chlorinated methane-respiring bacteria exists (Table 1.1). Mixed culture Dhb-CF contains a *Dehalobacter* population that grows by transforming CF to stoichiometric amounts of DCM and inorganic chloride (50). This culture generates DCM as end product and detoxification is not achieved; however, this finding demonstrates that bacteria that use chlorinated methanes as respiratory electron acceptors exist. Organohalide-respiring bacteria have revolutionized groundwater remediation at sites contaminated with chlorinated ethenes, and the discovery of a bacterium that respire CF to DCM holds promise that this activity is not uncommon and a broader group of bacteria uses CF as well as other chlorinated methanes as electron acceptors.

Table 1.1 Gibbs free energy changes associated with the reductive dechlorination of chlorinated methanes (26).

Chlorinated methane	$\Delta G_0'$ (kJ/mol H ₂)
CT	-192.6
CF	-170.8
DCM	-157.4
CM	-153.2

1.3.4 Remediation of chlorinated methanes

Extensive use and improper handling and disposal of chlorinated methanes have resulted in the release of large quantities of CT, CF and DCM into the environment. Once released, chlorinated methanes can reach aquifers and threaten ecosystem and human health. Remediation of subsurface environments impacted with chlorinated methanes is necessary to minimize exposure and the possible detrimental effects, but can prove to be challenging due to the inherent difficulties of subsurface monitoring. A number of physical and chemical groundwater remediation technologies have been developed and implemented with success at different contaminated sites, but given the complexity of subsurface environments and the common presence of contaminant mixtures, a combination of physical and chemical approaches with microbial-based technologies is often necessary (109).

1.3.4.1 Physical and chemical remediation technologies

Since the mid 1980s, pump-and-treat systems have become one of the most widely used technologies for groundwater remediation (38). Conventional pump-and-treat methods involve pumping contaminated water to the surface for treatment. Once at the surface, the contaminated water is remediated using one or a combination of the following methods: air stripping, activated carbon, ion exchange, reverse osmosis, chemical precipitation, chemically-assisted clarification, filtration, ultraviolet radiation oxidation and/or biological treatment (38). Pump-and-treat systems have been used primarily to control the movement of contaminated groundwater, preventing the continued expansion of the contaminated zone (i.e., hydraulic containment), and to reduce the dissolved contaminant concentrations in groundwater to levels that comply with the cleanup standards postulated by the regulatory agencies (i.e., treatment) (31). Amid the widespread use of pump-and-treat systems, reports and evaluations dating to the early 1990s started calling attention to the general failure of this approach to achieve the reduction of contaminants to levels required by health-based standards in 5 to 10 years, the time anticipated during the design phase of most projects (44, 51, 69, 89).

The limitations and concerns related to pump-and-treat systems have lead to an increased interest in more “aggressive” strategies for source zone remediation. In recent years, remediation practitioners have turned away from pump-and-treat systems and towards alternative *in situ* physical-chemical source zone remediation technologies including surfactant enhanced aquifer remediation (SEAR), cosolvent flushing, chemical oxidation (ISCO) and thermal treatment.

Surfactant enhanced aquifer remediation (SEAR) and cosolvent flushing are source zone removal technologies that utilize the injection of chemicals to solubilize contaminant source zones (24). Surfactants (e.g., soaps and detergents) and cosolvents (e.g., alcohols) can increase the aqueous phase solubility of contaminants allowing for the subsequent extraction of the contaminant mass (17). After making contact with the source zone material, the surfactant or cosolvent is flushed out, along with the solubilized contaminant mass, and collected above surface (24). One major disadvantage of utilizing surfactant or cosolvent flushing as a remediation strategy is that the contaminants are not transformed to non-toxic compounds *in situ*, therefore requiring additional processing once collected at ground level.

In situ chemical oxidation (ISCO) is another environmental remediation technique used to reduce the concentration of target contaminants in soil and/or groundwater. In field applications of ISCO, a strong chemical oxidant (e.g., Fenton's reagent, permanganate, persulfate or ozone) is delivered and distributed within the source zone (40). The reaction of the oxidant (or any reactive species produced by the oxidant in the subsurface environment) with the contaminant mass within the source zone can potentially produce benign end products (i.e., mineralization products such as carbon dioxide and inorganic chloride) (17, 40). In general, the factors controlling and limiting the successful implementation of ISCO as a source zone remediation strategy are: delivery, distribution and mixing of the oxidant with the contaminant mass, overcoming the natural oxidant demand, gas formation and emissions, losses due to permeability, and potential decreases in water quality due to increased metal mobility and decreased pH (62).

In situ thermal remediation technologies consist of directly injecting heat into the subsurface with the intent to destroy or mobilize and recover volatile organic contaminants (32). Thermal treatments now commonly used to remediate contaminant source zones are steam-enhanced extraction, electrical-resistance heating and thermal-conductive heating (22). The application of *in situ* thermal remediation technologies in several contaminant-impacted sites has achieved significant reductions in contaminant concentrations to very low levels; however, thermal treatment also has limitations (18, 32). The main limitations for thermal treatment are the potential for source zone mobilization into previously uncontaminated areas, potential formation of undesirable intermediates or degradation products and the high-energy costs associated with these technologies (21).

Although physical-chemical source zone remediation technologies can remove significant amounts of contaminant mass relatively quickly, these aggressive techniques infrequently reach the cleanup levels established by regulatory agencies (118). As a result, post-treatment source zone contaminant concentrations usually exceed health-based standards and can still pose risks to the environment and human health. Furthermore, physical-chemical remedies can result in detrimental changes to the distribution and/or the physical and chemical characteristics of the remaining contaminant mass, potentially making subsequent remediation more challenging (9, 97). Current research in the field of physical-chemical remediation technologies aims to address and overcome the limitations inherent to these remedies; however, alternative, passive remedial approaches, such as *in situ* bioremediation, are being used to replace or to follow physical-chemical remediation strategies.

1.3.4.2 *In situ* bioremediation

The capacity of microbial populations to degrade contaminants in subsurface environments can be enhanced either by stimulation of the indigenous microbiota by the addition of nutrients and/or electron acceptors/donors (i.e., biostimulation) or by bioaugmentation (116, 117). In principle, bioaugmentation is a remediation technology, in which the catabolically relevant organisms are added to kick-start or speed up contaminant cleanup *in situ* (120). Bioaugmentation of contaminated aquifers with dechlorinating consortia containing ethene-producing *Dehalococcoides mccartyi* strains has proven to be effective at achieving complete detoxification at chlorinated ethene-contaminated sites (27, 83, 94, 111). A similar bioremedial approach is desirable for the detoxification of sites impacted with chlorinated methanes.

1.3.4.2.1 Monitoring bioremediation processes in situ: molecular biological tools (MBTs)

Monitoring relevant microbial populations and assessing the fate of contaminants *in situ* is a key aspect of any successful application of bioremediation. In recent years, a variety of analytical and molecular biological tools (MBTs) that allow for direct or indirect monitoring of bioremediation processes have become available (7). One widely used tool for assessing changes in the size of a target bacterial population is the monitoring of 16S rRNA genes via quantitative real-time polymerase chain reaction (qPCR). 16S rRNA genes are commonly used to determine the phylogeny of organisms because they are among the most conserved genes across bacterial and archaeal taxa (126). Although 16S rRNA gene quantification has proven to be useful to monitor the size and distribution of populations of interest, it does not provide information about the metabolic activity of the target population(s) *in situ*. Thus, additional lines of evidence need to be

collected to ascertain that bioremediation of the target contaminant(s) is occurring as a result of the metabolic activity of a particular microbial population.

Since 16S rRNA gene-targeted analyses often fail to provide sufficient resolution to monitor the organisms that are directly involved in the metabolic transformation of the target contaminant, identification of alternative gene targets has become necessary. qPCR can also be applied to functional genes that code for proteins involved in contaminant transformation. For example, members of the *Dehalococcoides* genus can greatly differ on the type of chloroorganic electron acceptor(s) used for respiration, while still sharing a high degree of 16S rRNA gene similarity (> 98% identity). qPCR approaches targeting functional genes involved in the reductive dechlorination of specific chlorinated compounds have been employed to confirm *Dehalococcoides* strain purity and to monitor different *Dehalococcoides* strains simultaneously (110, 119). Currently, dechlorinating *Dehalococcoides mccartyi* strains are often monitored in groundwater by quantifying reductive dehalogenase (RDase) genes associated with specific transformation reactions (86). Bioremediation practitioners often use both phylogenetic (16S rRNA gene-based) and process-specific (functional) MBTs to obtain a better understanding of the microbial processes involved in contaminant detoxification *in situ*.

1.3.4.2.2 Monitoring bioremediation processes in situ: compound specific isotope analysis (CSIA)

Compound-specific isotope analysis (CSIA) is another technique that has become very useful to bioremediation practitioners in recent years. CSIA is a method that measures the ratios of naturally occurring stable isotopes in environmental samples. Every element that comprises the molecules of a given compound has a characteristic isotopic

signature (i.e., relative abundance of the heavy and light isotopes of each element within the compound) (29). The isotopic signature of a compound is directly related to and changes with its source and manufacturing process; therefore, the composition of stable isotopes contained in a compound can allow the identification of different sources of such compound (e.g., anthropogenic contamination versus natural background) (23). Although the heavy and light isotopes of an element are nearly identical, the differences in atomic mass result in dissimilar bond energetics (100). Chemical bonds involving light isotopes are easier to break than those containing the heavy isotopes of an element (100). As a compound is transformed or degraded, the remaining product becomes isotopically heavier – a process called isotopic fractionation (Figure 1.4). CSIA can give insights into the fate of target contaminants as remediation progresses by exploring the changes in the ratios of its stable isotopes during the course of biodegradation or other processes (64). Furthermore, elemental isotopic ratios are also a function of the degradation pathway. CSIA can potentially distinguish biodegradation from physical-chemical (i.e., abiotic) processes that lead to contaminant degradation. In addition, contaminant dilution, volatilization and evaporation can also be discriminated from transformation and/or degradation by employing CSIA.

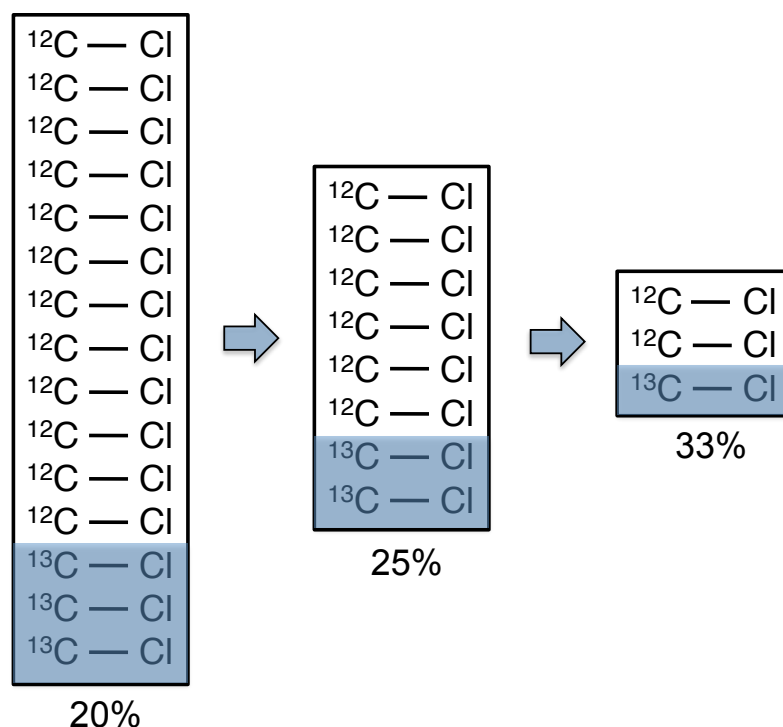


Figure 1.4 Illustration of heavy stable isotope enrichment (e.g., ^{13}C) during degradation. Image adapted from ITRC CSIA Fact Sheet (64).

Stable carbon isotope analysis, in particular, is the experimental determination of the proportion of a given stable carbon isotope (^{12}C and ^{13}C) in a sample. Gas chromatography-isotope ratio mass spectrometry (GC-IRMS), or the highly sensitive gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS), can be used to measure the stable carbon isotope ratios averaged over the bulk of a compound (including all carbon atoms). For the analysis of stable carbon isotope ratios specifically, samples are first converted to CO_2 gas and then subjected to IRMS. The IRMS monitors ions with mass to charge ratios of 44, 45 and 46, which correspond to the ions produced from CO_2 molecules containing ^{12}C and ^{13}C in various combinations with the different isotopes of oxygen (14). The bulk carbon isotope ratios obtained from such analyses are reported as difference in per mil ($\text{‰}^{13}\text{C}$) with respect to an international reference

standard (29). The Peedee Belemnite international carbon standard has a relative isotope abundance of 98.89/1.11 $^{12}\text{C}/^{13}\text{C}$, revealing that about 99% of all carbon on earth consists of the stable carbon isotope ^{12}C and approximately 1% of the stable isotope ^{13}C (57, 100). Once the stable carbon isotope ratio is measured, the Rayleigh equation can be used to describe isotopic fractionation during contaminant degradation (95, 108):

$$\ln((1000+\delta^{13}\text{C}_0+\Delta\delta^{13}\text{C})/(1000+\delta^{13}\text{C}_0)) = (\epsilon_{\text{bulk}}/1000)\ln(f)$$

where $\delta^{13}\text{C}_0$ is the carbon isotope composition of the compound at time zero, $\Delta\delta^{13}\text{C}$ is the change in the carbon isotope composition from time zero to time t, ϵ_{bulk} is the bulk carbon isotope enrichment factor, and f is the molar fraction of the compound remaining at time t. The Rayleigh equation is commonly used and has proven to be extremely valuable because it relates difference in bulk isotope ratios directly to changes in contaminant concentration (29). Consequently, stable carbon isotope ratios can be used as a monitoring tool to quantify how much degradation has occurred *in situ* (29).

CSIA, and more specifically stable carbon isotope analysis, has been successfully implemented to demonstrate and monitor *in situ* bioremediation of target contaminants (100). For example, in a study performed by Sherwood Lollar et al., stable carbon isotope measurements were used to evaluate the effectiveness of natural attenuation of chlorinated ethene contamination in groundwater (115). CSIA measurements confirmed that tetrachloroethene (PCE) and trichloroethene (TCE) dissolved in the anoxic portions of the plume had an isotopic enrichment in ^{13}C consistent with the effects of intrinsic biodegradation (115). This study was the first to provide definitive evidence for the reductive dechlorination of chlorinated hydrocarbons *in situ* (115). Stable carbon isotope fractionation was also utilized to quantify the extent of microbial *in situ* transformation in an aquifer contaminated with benzene and toluene (124). The data collected by Vieth et

al. were used to estimate *in situ* degradation rates of benzene and toluene, and to develop conceptual models required for evaluating further remediation approaches at the site (124).

On the subject of chlorinated methanes, Heraty et al. and Nickolausz et al. explored stable carbon isotope fractionation during the degradation of DCM by methylotrophic bacteria (55, 106). The stable carbon isotope fractionation factors obtained for several known DCM-degraders under oxic and nitrifying conditions were all in the same order of magnitude (106). In other words, variations in 16S rRNA gene-based phylogeny, amino acid sequence of the different glutathione-dependent DCM dehalogenases or electron acceptor conditions did not translate into distinct fractionation of the stable carbon isotopes in DCM (106). Based on the results of their studies, the authors concluded that the use of stable carbon isotope fractionation factors for the assessment of the degradation of DCM by methylotrophic bacteria is therefore justified under oxic as well as nitrifying conditions (106).

The isotopic fractionation data collected via CSIA during the transformation of a target contaminant, along with the calculation of kinetic isotope effects (KIEs), can be used to derive information about the transformation mechanisms. KIEs can help to identify degradation pathways by exploring the theoretical rate change due to isotopic substitution at a site of bond breaking or bond making in the rate-determining step of a mechanism (i.e., primary isotope effects) (29). The computation of secondary isotope effects – rate change due to isotopic substitution at a site other than that of bond breaking or bond making – can give additional insights into a contaminant's transformation mechanism by accounting for differences in isotopic fractionation due to

steric hindrance or inductive effects (i.e., electronegativity) (29). KIE values are normalized for the presence of non-reactive and indistinguishable reactive sites and can be calculated according to:

$$KIE = 1/(1+(z*\epsilon_{\text{reactive}}/1000))$$

where z is the number of indistinguishable reactive sites and a correction factor for the effects of intramolecular competition and $\epsilon_{\text{reactive}}$ is the reactive position-specific enrichment factor. $\epsilon_{\text{reactive}}$ can be determined according to:

$$\ln((1000+\delta^{13}C_0+(n/x)\Delta\delta^{13}C)/(1000+\delta^{13}C_0)) = (\epsilon_{\text{reactive}}/1000)\ln(f)$$

where n is the number of carbon atoms in the molecule and x is the number of carbon atoms in the reactive position. Theoretical KIEs calculated for all possible transformation mechanisms of the target contaminant can be compared against the isotopic fractionation values obtained during *in situ* transformation. A KIE value matching the stable isotope fractionation observed *in situ* can reveal the dominant transformation mechanism for a specific contaminant of interest. Several studies have successfully applied CSIA and the calculation of KIEs to determine reaction mechanisms (28, 43, 56, 60, 63, 123). Thus, CSIA provides both a tool to monitor contaminant degradation *in situ*, and an approach to elucidate the pathway(s) and mechanism(s) controlling contaminant transformation or degradation at impacted field sites.

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CHAPTER 2

DICHLOROMETHANE FERMENTATION BY A *DEHALOBACTER* SP. IN AN ENRICHMENT CULTURE DERIVED FROM PRISTINE RIVER SEDIMENT

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2.1 Abstract

Dichloromethane as the sole substrate supported growth of a *Dehalobacter* sp. in an enrichment culture derived from non-contaminated river sediment. DCM was not reductively dechlorinated and acetate was produced, indicating DCM fermentation and further suggesting *Dehalobacter* growth is not limited to organohalide respiration.

2.2 Introduction

The chlorinated methanes carbon tetrachloride (CT), chloroform (CF), and dichloromethane (DCM) are common groundwater contaminants (23). No microbes capable of metabolizing CT have been reported, but the interaction of CT with electron transfer-active biomolecules can yield the trichloromethyl radical, which leads to unspecific transformation reactions and consequently, CF formation (22). CF can be co-metabolically transformed by several sulfate-reducing and methanogenic enrichment cultures (11, 30), and a recent report demonstrated growth of a *Dehalobacter* sp. linked to reductive dechlorination of CF to DCM and inorganic chloride as end products (8). This finding suggested that CF organohalide respiration provides an additional source of

DCM in anoxic environments. Under oxic conditions, DCM is readily degraded by methylotrophic bacteria harboring glutathione-dependent DCM dehalogenases (3, 15, 29); however, the fate of DCM in anoxic habitats is poorly understood. A few studies have demonstrated DCM degradation under denitrifying (7), acetogenic (5, 19) and methanogenic (6) conditions and DCM fermentation was observed in a packed-bed digester sludge reactor (4). The latter study suggested that methanogens, sulfate reducers, and nitrate reducers were not directly responsible for DCM degradation. To date, *Dehalobacterium formicoaceticum* is the only organism described to anaerobically metabolize DCM via a fermentative pathway to acetate, formate, and inorganic chloride (18, 20).

Dehalobacter spp. are characterized as strictly organohalide-respiring organisms, and no other growth-supporting substrates or substrate combinations could be identified (21, 26). *Dehalobacter* spp. respire chlorinated ethenes (14), chlorinated ethanes (9, 27), 4,5,6,7-tetrachlorophthalide (31), β -hexachlorocyclohexane (28), and CF (8), and have recently been implicated in reductive dechlorination of dichlorobenzenes and monochlorobenzene (24).

2.3 Methods, Results and Discussion

Pristine freshwater sediment was collected from Rio Mameyes in Luquillo, Puerto Rico in October 2009 (latitude 18°21'43.9", longitude -65°46'10"). Microcosms were established as described (13) with the following modifications: A pipettable slurry (0.2 g [dry weight] mL⁻¹) was prepared by mixing the sediment with anoxic, bicarbonate-buffered (30 mM, pH 7.2) and HEPES-buffered (10 mM, pH 7.2) mineral salts medium (17). Inside an

anoxic chamber (Coy, Ann Arbor, MI) filled with H_2/N_2 (3%/97%, vol/vol), 12-mL aliquots were dispensed into sterile 24-mL (nominal capacity) glass vials. Each microcosm received 20 mg L^{-1} DCM ($\approx 128\text{ }\mu\text{M}$ aqueous phase concentration) and triplicate microcosms were incubated statically at room temperature in the dark. DCM and chloromethane (CM) were monitored via manual headspace injections (0.1 mL) with a Hewlett-Packard (HP) 6890 gas chromatograph (GC) equipped with an HP-1 Crosslinked Methyl Siloxane column (30 m x 0.32 mm; film thickness, 0.25 μm nominal) and an electron capture detector (ECD). The GC inlet was maintained at $235\text{ }^\circ\text{C}$ and 4.98 psig with a total helium flow of 73.6 mL min^{-1} , and the inlet split ratio was 10:1. The oven temperature for the GC was kept at $35\text{ }^\circ\text{C}$ for 5.50 minutes followed by an increase at a rate of $25\text{ }^\circ\text{C min}^{-1}$ until the oven reached $145\text{ }^\circ\text{C}$. The column was operated in the constant flow mode at 1.0 mL min^{-1} and 4.98 psig. The ECD was operated at a temperature of $235\text{ }^\circ\text{C}$ with nitrogen as makeup gas at a flow rate of 30 mL min^{-1} . The method provided a linear detector response for DCM and CM concentrations from $1.5\text{ }\mu\text{M}$ to $385\text{ }\mu\text{M}$ and from $85\text{ }\mu\text{M}$ to $3,415\text{ }\mu\text{M}$, respectively. Standards were prepared by adding known amounts of DCM or CM to culture vessels containing medium. Methane, acetate, and formate were monitored as described by Amos et al. (1) and He et al. (12).

After 4 weeks of incubation, less than 10% of the initial amount of DCM disappeared from the autoclaved control microcosms, but the initial dose of DCM was consumed in live microcosms. Nine additional DCM doses of 20 mg L^{-1} were consumed at increasing rates. Serial transfer cultures (4% inocula, vol/vol) in 160 mL glass serum bottles containing 96 mL of medium received five to 10 DCM feedings of 20 mg L^{-1} ($\approx 150\text{ }\mu\text{M}$ aqueous phase concentration), and after three transfers, sediment-free enrichment cultures were obtained. With DCM as the sole substrate, visible turbidity developed after

five DCM feedings. A 10th transfer culture fed approximately 125 mg L⁻¹ DCM (≈950 μM aqueous phase concentration) was characterized in more detail. The culture consumed DCM at a rate of 4.0 mg L⁻¹ per day and tolerated at least 200 mg L⁻¹ without apparent inhibition. Acetate, methane, and biomass were products of DCM degradation (Figure 2.1); formate was not detected. CM was never detected and efforts to grow the culture with CM were not successful (i.e., CM persisted in the culture medium) suggesting that the culture degrades DCM but not CM.

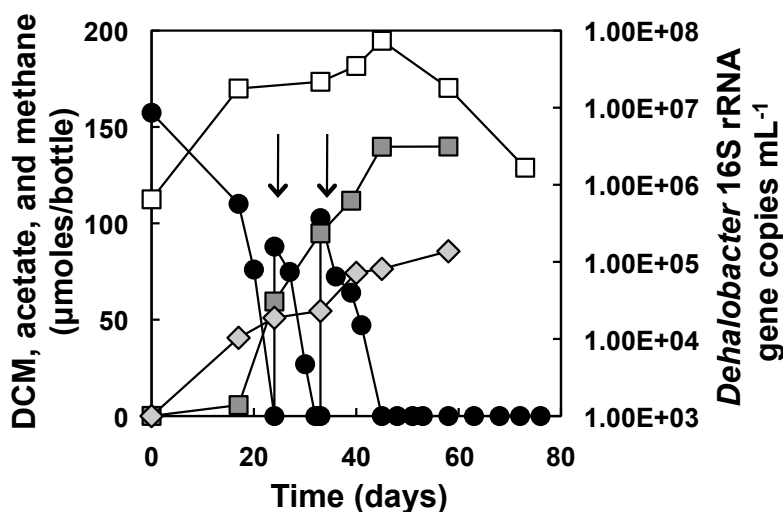


Figure 2.1 DCM degradation in the Rio Mameyes enrichment culture. DCM degradation (solid black circles) coincided with acetate formation (solid light gray diamonds) and an increase in *Dehalobacter* 16S rRNA gene copy numbers (open squares). Methane formation (solid gray squares) started following acetate production. The culture received two additional DCM feedings as indicated by the arrows. Replicate cultures exhibited similar degradation and product formation patterns.

To explore the role of methanogens in the enrichment culture, the effects of 40 mg L⁻¹ CF (\approx 228 μ M aqueous phase concentration) and 1 mM 2-bromoethanesulfonate (2-BES), both known methanogenesis inhibitors (2, 16), on methane formation and DCM degradation were monitored (Figure 2.2). CF inhibited both DCM degradation and methane formation. This inhibition persisted even with lower CF concentrations of 5 mg L⁻¹ suggesting high susceptibility of the DCM-degrading population(s) to CF. The addition of 2-BES prevented methane formation while DCM degradation occurred, albeit at 2.2 fold lower rates. Following the 2-BES treatment, methane production was abolished and DCM was degraded at rates comparable to those observed in the methanogenic culture. These observations suggest unspecific inhibition by 2-BES, as was observed previously (16), and indicated that methanogens were not involved in DCM degradation. DCM fermentation in the non-methanogenic culture yielded acetate and biomass. The attained partial mass balance suggests a 2:1 DCM:acetate ratio and concurs with a previous report on DCM fermentation in an acetogenic mixed culture (19).

To investigate the populations involved in DCM degradation, DNA was extracted and a bacterial 16S rRNA gene clone library was constructed using the Invitrogen pCR2.1-TOPO cloning kit (Invitrogen, Carlsbad, CA) as described (25, 32). DNA was obtained from 5 mL of the 10th transfer culture suspension using the QIAmp DNA Micro Kit (Qiagen, Germantown, MD) for the isolation of genomic DNA from the pelleted cell material with the following modifications: Four μ L of RNase A (100 mg mL⁻¹) were added to 200 μ L Buffer AL and the samples were incubated at 70 °C for 10 minutes. Out of 208 colonies screened, 105 carried a 1,500 bp long 16S rRNA gene insert, 81 of which were identified as *Dehalobacter* sequences. Six randomly chosen *Dehalobacter* positive clones yielded nearly full-length (1,480 bp) sequences (GenBank accession numbers

JN900241-246) and all shared 97% identity with clone sequence CK10 (1333/1381 positions; GU320656) and 95% identity (1352/1421 bases) with *Dehalobacter* sp. 1,1-DCA1 (DQ777749) (Figure 2.3). It is currently unclear if these sequences represent one or more *Dehalobacter* strains; however, insights from genome sequencing revealed that *Dehalobacter* sp. strain MS carries three 16S rRNA gene copies (E. A. Edwards, personal communication). The predominance of *Dehalobacter* 16S rRNA gene fragments in the clone library suggested that one or more *Dehalobacter* strains were involved in DCM degradation. Of the 24 clones with non-*Dehalobacter* 16S rRNA gene inserts, about half of the fragments resembled *Acetobacterium* sp. sequences, and the remainder had highest nucleotide sequence identities to environmental clone sequences without cultured representatives.

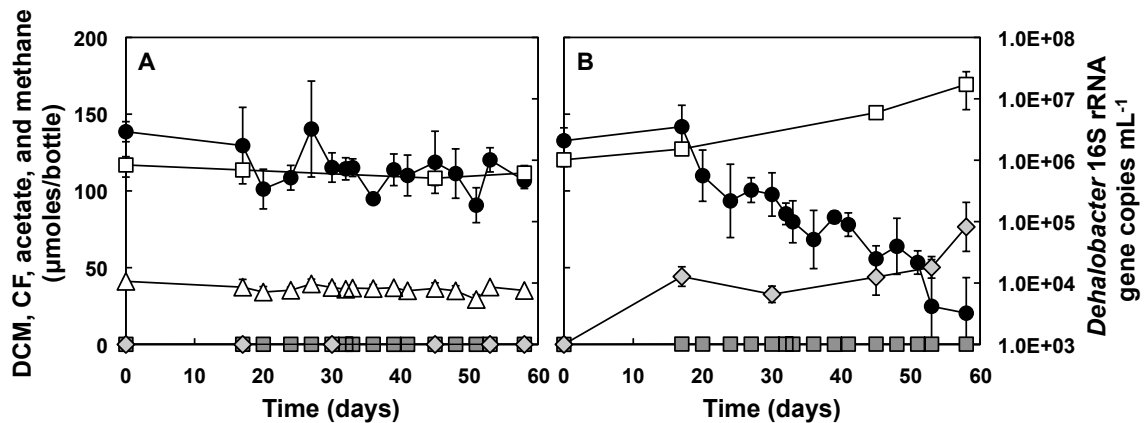


Figure 2.2 Effects of the methanogenesis inhibitors chloroform (40 mg L⁻¹) and 2-BES (1 mM) on the DCM-degrading enrichment culture. Panel A demonstrates that chloroform (open triangles) was not transformed and prevented DCM degradation (solid black circles), methane formation (solid gray squares), acetate formation (solid light gray diamonds), and the increase of *Dehalobacter* 16S rRNA genes (open squares). Panel B demonstrates that 1 mM 2-BES inhibited methane formation but did not prevent DCM degradation, acetate formation, or *Dehalobacter* growth.

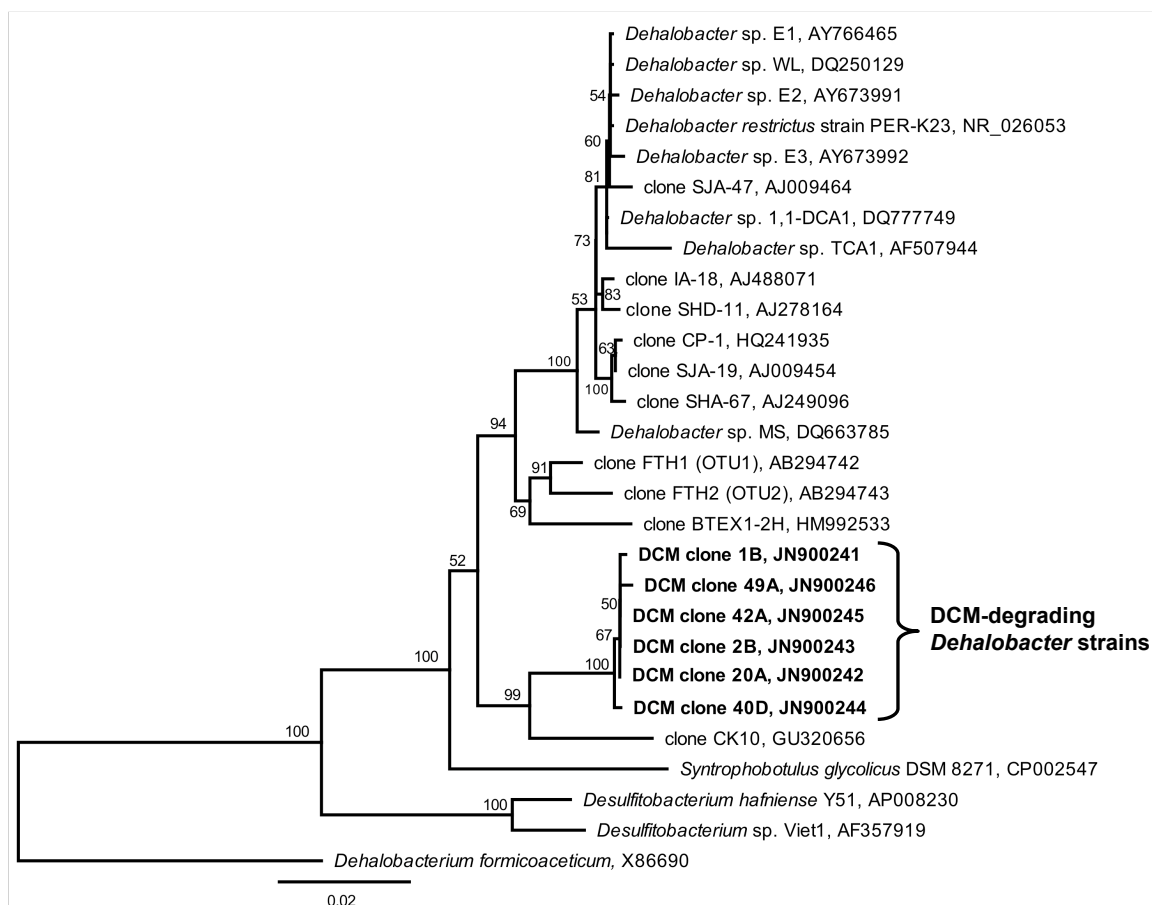


Figure 2.3 16S rRNA gene-based phylogenetic tree demonstrating the affiliation of the DCM-degrading enrichment clones with the *Dehalobacter* genus. The tree is based on 1,194 fully aligned bases to accommodate incomplete sequences of *Dehalobacterium formicoaceticum* (lacked a portion of the 5' end) and several environmental clones. An alignment of the available, nearly complete 16S rRNA gene sequences did not change the tree topology. The alignments were generated using Clustal W with the BLOSUM cost matrix, a gap open cost of 10 and gap extension cost of 3. The tree algorithm uses the HKY (Hasegawa, Kishino, Yano) Genetic Distance Model and Neighbor Joining with 1,000 Bootstrap iterations. Only branches with > 50% bootstrap support are shown. *Dehalobacterium formicoaceticum* is the outgroup. The scale bar indicates substitutions per site (0.02 = 2/100 bp).

To conclusively demonstrate growth of *Dehalobacter* in the DCM-degrading culture, the abundance of *Dehalobacter* 16S rRNA gene copies was measured using a quantitative real-time PCR approach (qPCR). An established qPCR protocol (10) was used with the following modifications: qPCR was conducted using an ABI 7500 Fast Real Time PCR System equipped with SDS v2.0.3 software using the default SYBR Green cycling parameters and the 2x Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Calibration was performed in triplicate with serial 10-fold dilutions of a *Dehalobacter* 16S rRNA gene-containing plasmid from approximately 3.37×10^7 copies down to approximately 3 copies. Standard curves demonstrated a slope of -3.4, a y-intercept of 38 and an R^2 value of 0.99. As shown in Figure 1.1, the *Dehalobacter* 16S rRNA gene copy numbers increased to 7.38×10^7 mL⁻¹ of culture suspension concomitant with DCM consumption, yielding $(2.91 \pm 1.13) \times 10^5$ *Dehalobacter* 16S rRNA gene copies per μ mol of DCM consumed. When DCM feedings were stopped, the *Dehalobacter* 16S rRNA gene copy numbers decreased to about 10^5 cells mL⁻¹ (Figure 1.1) but increased again to about 10^7 cells mL⁻¹ when additional DCM was provided (not shown). No growth was observed and *Dehalobacter* 16S rRNA gene copies did not increase in cultures that received no DCM. This is not surprising because DCM was the sole substrate added and only negligible amounts (< 0.5 mg L⁻¹) were introduced with the inoculum. In vessels amended with hydrogen (80% of the headspace) instead of DCM, acetate was formed confirming that bacteria capable of CO₂ reductive acetogenesis were present (as indicated by the clone library results); however, the hydrogen-fed culture lost its ability to degrade DCM. While DCM degradation occurred in both bicarbonate- or HEPES-buffered microcosms, DCM degradation by the sediment-free enrichment culture only occurred in medium amended with CO₂, similar to what has been observed for *Dehalobacterium formicoaceticum* (20). *Dehalobacter* 16S rRNA

gene copies did not increase without DCM indicating that the DCM degrader did not grow via CO₂ reductive acetogenesis. A recent report demonstrated that *Dehalobacter* sp. strain MS couples CF-to-DCM reductive dechlorination with growth (i.e., organohalide respiration) (8), but the DCM-degrading culture failed to reductively dechlorinate CF suggesting that distinct *Dehalobacter* populations are responsible for CF reductive dechlorination and DCM fermentation. Strain MS produced 2.5×10^7 *Dehalobacter* 16S rRNA gene copies per μmol of Cl⁻ released from CF dechlorination to DCM. The DCM-degrading culture produced about two orders of magnitude fewer *Dehalobacter* 16S rRNA gene copies (i.e., $1.45 \pm 0.56 \times 10^5$ per μmol of Cl⁻ released), suggesting that DCM fermentation is energetically less favorable than organohalide respiration. These findings indicate that members of the genus *Dehalobacter* have the ability to degrade CF and DCM to innocuous products. The CF-respiring *Dehalobacter* sp. tolerated at least 100 mg L⁻¹ CF but CF dechlorination was inhibited when DCM accumulated to 2 mM (≈ 170 mg L⁻¹ aqueous phase concentration) (8). The DCM-degrading culture tolerated at least 200 mg L⁻¹ DCM, and it will be interesting to explore if a mixed consortium containing the CF-to-DCM-respiring *Dehalobacter* sp. and the DCM-fermenting *Dehalobacter* sp. will efficiently detoxify CF. If successful, such a consortium should be tested as a bioaugmentation inoculum to initiate chlorinated methane degradation at field sites where CF and DCM persist.

To date, *Dehalobacterium formicoaceticum* has been the only described anaerobe capable of DCM fermentation (20), and our findings expand the diversity of microbes that share this trait. Furthermore, the discovery of a DCM-fermenting *Dehalobacter* sp. demonstrates that *Dehalobacter* metabolism is not restricted to organohalide respiration. This observation has implications for contaminated site monitoring, and the increase in

the *Dehalobacter* population size should not be linked to reductive dechlorination processes without detailed analysis of the contaminant transformation pathways (e.g., achieved through the analysis of dehalogenase genes associated with contaminant transformation) and products.

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CHAPTER 3

BIOAUGMENTATION WITH *DEHALOBACTER*-CONTAINING CONSORTIA ACHIEVES COMPLETE CHLOROFORM DETOXIFICATION IN ANOXIC MICROCOSMS

Reproduced in part with permission from Justicia-Leon, S. D.; Mack, E. E.; Griffiths, D. R.; Tang, S.; Edwards, E. A.; Löffler, F. E. Bioaugmentation with *Dehalobacter*-containing consortia achieves complete chloroform detoxification in anoxic microcosms. *Environ. Sci. Technol.* 2012, submitted for publication. Unpublished work copyright 2012, American Chemical Society.

3.1 Abstract

Chloroform (CF) is a widespread groundwater contaminant not susceptible to aerobic degradation. Under anoxic conditions, CF can undergo abiotic and co-metabolic transformation but detoxification is generally not achieved. The recent discovery of distinct *Dehalobacter* strains that respire CF to dichloromethane (DCM) and ferment DCM to non-chlorinated products promises that bioremediation of CF plumes is feasible. To explore if the CF-to-DCM-dechlorinating culture Dhb-CF and the DCM-degrading consortium RM promote CF detoxification, anoxic microcosms established with aquifer material from a CF-contaminated site were augmented with both the CF-dechlorinating and DCM-degrading consortia. Microcosms that received 6% (vol/vol) of the CF-to-DCM-dechlorinating culture Dhb-CF to achieve an initial *Dehalobacter* cell titer of $1.56 \pm 0.88 \times 10^4 \text{ mL}^{-1}$ dechlorinated CF to stoichiometric amounts of DCM. Subsequent augmentation with 3% (vol/vol) of the DCM-degrading consortium RM to an initial *Dehalobacter* cell abundance of $1.15 \pm 0.17 \times 10^2 \text{ mL}^{-1}$ achieved complete DCM degradation in microcosms amended with 10 mM bicarbonate. Growth of the CF-respiring and the DCM-degrading *Dehalobacter* populations and detoxification were also observed in microcosms that received both inocula simultaneously, suggesting that

anaerobic bioremediation (e.g., bioaugmentation) is a viable remedy at CF- and DCM-contaminated sites.

3.2 Introduction

Chloroform (CF) has had a multitude of applications since its discovery in the 1830s (4). CF was used as an inhaled anesthetic, an extraction solvent, an intermediate for the production of refrigerants and other chemicals, as a heat transfer agent in fire extinguishers, and as a fumigant (2). Currently, CF is mainly used as a reagent for the synthesis of monochlorodifluoromethane (HCFC-22), a widely used refrigerant that will soon be phased out under the Montreal Protocol due to its ozone depleting potential and status as a potent greenhouse gas (54). Most CF entered the environment as a consequence of improper handling, storage and disposal practices. Additional sources include drinking and wastewater chlorination processes, which produce some CF, and the release from natural sources (34, 45). In addition, the abiotic and biotic transformation of carbon tetrachloride (CT) contributes to CF formation at contaminated sites (33, 50). CF has been found in over 717 of the 1,430 National Priority List sites throughout the United States and its territories (2, 17). Human exposure to CF is of concern due to detrimental health effects, and the U.S. EPA considers CF as a probable human carcinogen (15). A Maximum Contaminant Level (MCL) of total trihalomethanes (i.e., CF, bromoform, bromodichloromethane and dibromochloromethane) of 80 parts per billion in drinking water has been established (16). CF is also toxic to microbes and inhibits microbial processes including methanogenesis and the reductive dechlorination of chlorinated ethenes (5, 6, 11).

CF is recalcitrant and persists in many groundwater aquifers because the contaminant is not susceptible to metabolic or growth-linked transformation under oxic conditions.

Transformation of CF as a result of abiotic and co-metabolic anaerobic processes has been reported, but these reactions are generally slow and difficult to manipulate *in situ* (7, 8, 26-28, 32, 51, 56). Recently, *Dehalobacter* strains capable of organohalide respiration of CF to DCM were identified in two distinct mixed cultures (23, 35). Dhb-CF, a subculture derived from a 1,1,1-trichloroethane-dechlorinating enrichment culture, produces DCM as end product indicating that the *Dehalobacter* strain(s) in this culture cannot degrade DCM (23).

DCM is also toxic and a human health hazard, and the U.S. EPA has set a drinking water MCL of 5 parts per billion (1, 16). DCM is readily degraded under oxic conditions by methylotrophic bacteria and the biochemistry of this process is well understood; however, CF reductive dechlorination occurs in anoxic environments, and little is known about the fate of DCM in the absence of oxygen (37, 55). To date, only two types of microorganisms have been described to anaerobically metabolize DCM –

Dehalobacterium formicoaceticum and distinct *Dehalobacter* populations identified in two DCM-degrading enrichment cultures (31, 35, 42, 43). Consortium RM was derived from pristine Rio Mayemes sediment and harbors *Dehalobacter* sp. strain RM1 capable of growth by fermenting DCM to acetate and inorganic chloride (31). Distinct *Dehalobacter* populations were implicated in CF reductive dechlorination and successive DCM “dehalofermentation” in an enrichment culture derived from subsurface soil (35). Growth of the DCM-degrading *Dehalobacter* strains in the two reported consortia was linked to DCM fermentation to non-chlorinated products (31, 35). These findings indicate that distinct *Dehalobacter* populations are involved in the reductive dechlorination of CF to

DCM and the dechlorofermentation of DCM to innocuous products, and that their combined activities may contribute to CF and DCM detoxification under anoxic conditions.

Field studies have demonstrated the effectiveness of augmenting contaminated aquifers with dechlorinating consortia containing ethene-producing *Dehalococcoides mccartyi* strains to achieve complete detoxification of chlorinated ethenes (14, 38, 44, 49). Prior to field applications of bioaugmentation consortia, laboratory-based microcosm treatability studies are essential to assess and predict the performance of such a strategy for *in situ* bioremediation. To explore the feasibility of achieving CF detoxification with the Dhb-CF and the RM consortia, a microcosm treatability study was conducted with aquifer materials collected from a CF-contaminated site.

3.3 Materials and Methods

3.3.1 Chemicals. CF (>99.8%) and DCM (>99.8%) were purchased from Acros Organics (Thermo Fisher Scientific, Fairlawn, NJ) and Sigma-Aldrich Co. (St. Louis, MO), respectively. All of the other chemicals used were reagent grade or better, unless otherwise specified.

3.3.2 Bioaugmentation cultures. Culture Dhb-CF was maintained without agitation at room temperature in defined, anoxic, reduced mineral salts medium buffered with 10 mM potassium phosphate (pH 7.2) and amended with CF (419 μM or 50 mg L^{-1} , aqueous phase concentration) as electron acceptor and lactate (5 mM) and hydrogen (4.1 mM, nominal concentration) as electron donors (40). CF and DCM concentrations were

monitored and culture Dhb-CF received additional CF upon complete transformation of CF to DCM. The culture vessels were purged with N₂ gas when DCM reached 200 mg L⁻¹ aqueous phase concentration to avoid DCM toxicity (23). Consortium RM was maintained without agitation at room temperature in defined, anoxic, reduced mineral salts medium containing 30 mM sodium bicarbonate (NaHCO₃) and 10 mM HEPES (pH 7.2) in a vessel containing a N₂/CO₂ (80%/20%, vol/vol) mixture as headspace (40). Consortium RM vessels were amended with DCM (589 µM or 50 mg L⁻¹, aqueous phase concentration), and received additional DCM feedings when DCM was depleted. Both consortium Dhb-CF and consortium RM were inhibited by CT and *Dehalobacter* sp. strain RM1 required the presence of bicarbonate to efficiently degrade DCM (31, 35).

3.3.3 Site description and sample collection. Soil and groundwater samples impacted with CF and DCM were collected from a contaminated site in California, USA, in December 2010. The site was in operation since the late 1950s, at a time before adequate industry practices to prevent spills and releases had been implemented. To control and remediate a large CT plume, a zero-valent iron Permeable Reactive Barrier (PRB) was installed. Quarterly PRB performance monitoring demonstrated CT removal ranging from 58-90% across three transects; however, CF and DCM concentrations increased downgradient of the PRB. A split-spoon sampler equipped with brass liners was used to collect soil near a monitoring well located directly downgradient of the PRB. Undisturbed soil cores were capped and sealed at both ends and shipped, along with 4 L of monitoring well groundwater, in a cooler with blue ice via overnight carrier to the laboratory. Microcosms were set up immediately upon arrival of the materials.

3.3.4 Medium preparation and microcosm setup. Reduced, defined, anoxic mineral salts medium was prepared as described, except that 10 mM potassium phosphate (pH 7.2) replaced the NaHCO_3 buffer (40). Inside an anoxic chamber (Coy, Ann Arbor, MI) filled with H_2/N_2 (3%/97%, vol/vol), pipettable slurries ($0.1 \text{ g [dry weight] mL}^{-1}$) were prepared by mixing site aquifer material with the phosphate-buffered mineral salts medium or with anoxic site groundwater. Approximately 100 mL of slurry were transferred to 160-mL glass serum bottles (nominal capacity). All bottles were capped with Teflon-lined, gray butyl rubber septa (West Pharmaceuticals, Lionville, PA) and amended with lactate (2 mM) and hydrogen (4.1 mM, nominal concentration) as electron donors. Glass syringes (Hamilton GASTIGHT® Liquid 10 μL Luer Lock Type 1701LT) were used to add undiluted DCM and/or CF to final aqueous phase concentrations of $372 \mu\text{M}$ ($\sim 30 \text{ mg L}^{-1}$) and $228 \mu\text{M}$ ($\sim 25 \text{ mg L}^{-1}$), respectively. Half of the microcosms received only CF, while the remaining microcosms were amended with both CF and DCM. After 2 days of equilibration, microcosms were augmented with 6% (vol/vol) of the CF-to-DCM dechlorinating culture Dhb-CF containing $2.59 \pm 1.46 \times 10^5$ *Dehalobacter* cells mL^{-1} to achieve an initial *Dehalobacter* cell abundance of $1.56 \pm 0.88 \times 10^4 \text{ mL}^{-1}$ in the microcosms. The DCM-degrading consortium RM served as inoculum (3% [vol/vol] containing $1.15 \pm 0.17 \times 10^4$ *Dehalobacter* cells) for one set of microcosms at the start of the experiment and to a replicate set of microcosms after all CF had been completely transformed to DCM by culture Dhb-CF. The inoculation achieved initial *Dehalobacter* sp. strain RM1 titers of $1.15 \pm 0.17 \times 10^2$ cells mL^{-1} in the microcosms. Heat-killed (i.e., autoclaved) and abiotic (no inocula) incubations served as negative controls. Triplicate microcosms per treatment were incubated without agitation at room temperature in the dark. Microcosms that depleted the initial CF dose received additional CF ($200 \mu\text{M}$ or $\sim 22 \text{ mg L}^{-1}$, aqueous phase concentration) unless otherwise specified. Microcosms that

had completed CF-to-DCM transformation and had been inoculated with the DCM-dechlorinating RM consortium, as well as the heat-inactivated and non-inoculated controls, received 10 mM NaHCO₃ to ensure that the CO₂ requirement of strain RM1 was met. Cultures that had consumed all DCM received additional DCM (372 µM or ~30 mg L⁻¹, aqueous phase concentration). The time required for CF and DCM consumption to occur in the microcosm experiments is reported as the average number of days ± standard deviation and reflects lag time variations between replicate microcosms.

3.3.5 Analytical methods. CF, DCM and chloromethane (CM) were monitored via manual headspace injections (100 µL) into a Hewlett-Packard (HP) 6890 gas chromatograph (GC) equipped with an electron capture detector (ECD) as described except for the following modifications: The GC inlet was maintained at a split ratio of 70:1 and the ECD was operated using nitrogen as makeup gas at flow rate of 60 mL min⁻¹ (31). The method provided a linear detector response for CF, DCM and CM concentrations from 34 µM - 662 µM, 24 µM - 495 µM and from 396 µM - 3500 µM (aqueous phase concentrations), respectively. Standards were prepared by adding known amounts of CF, DCM or CM to culture vessels containing medium and aqueous phase concentrations were calculated using published dimensionless Henry's law constants: 0.150 for CF, 0.0895 for DCM, and 0.361 for CM (22). Methane was monitored as described by Amos et al. (3).

3.3.6 DNA isolation and 16S rRNA gene quantification. To monitor the *Dehalobacter* populations responsible for CF-to-DCM reductive dechlorination and DCM degradation, genomic DNA was obtained from 1 mL microcosm suspension samples. DNA was extracted using the QIAmp DNA Mini Kit (Qiagen, Germantown, MD) for the isolation of

genomic DNA from the solids obtained following centrifugation in 1.5-mL plastic tubes at 16,000 x g for 15 min at room temperature. The abundances of 16S rRNA gene copies from the CF-to-DCM-respiring *Dehalobacter* strain and the DCM-degrading *Dehalobacter* sp. strain RM1 were measured using quantitative real-time PCR (qPCR). Primer Express v3.0 software was used to design primer/probe combinations that differentially amplified a 16S rRNA gene fragment of the CF-to-DCM-respiring *Dehalobacter* strain or strain RM1. For primer and probe design purposes, *Dehalobacter* 16S rRNA gene sequences were retrieved from the NCBI-nr database and aligned using ClustalW in MEGA v5.0 software package. The specificity of the primers and probes were evaluated using BLAST analysis and verified experimentally with genomic DNA from the CF-to-DCM-respiring culture Dhb-CF, *Dehalobacter restrictus* (DSM 9455), and from the DCM-degrading consortium RM containing *Dehalobacter* sp. strain RM1. The primers and probes targeting the 16S rRNA gene of the CF-to-DCM-respiring *Dehalobacter* strain in culture Dhb-CF and of the DCM-fermenting *Dehalobacter* sp. strain RM1 are shown in Table 3.1. qPCR was conducted using an ABI 7500 Fast Real Time PCR System equipped with SDS v2.0.3 software (Applied Biosystems, Carlsbad, CA). Every 20 μ L-reaction contained 10 μ L of 2x Taqman® Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA), 2 μ L of DNA template, and forward and reverse primers and probe at final concentrations of 300 nM each. The following thermocycling program was used: initial denaturation for 2 min at 50 °C and 10 min at 95 °C and 45 cycles of denaturation at 95 °C for 15 sec and annealing and extension at 60 °C for 1 min. Calibration used standard curves based on triplicate qPCR series obtained with 10-fold dilutions of the 16S rRNA gene of the CF-to-DCM-respiring *Dehalobacter* strain and the DCM-fermenting *Dehalobacter* strain. For this purpose, the 16S rRNA genes of both *Dehalobacter* strains were cloned in *E. coli* as described and

added as template DNA to yield reactions spanning a concentrations range from 3.38×10^7 copies down to approximately 3 copies (48).

3.4 Results

3.4.1 Detection, differentiation and quantification of the CF-to-DCM-respiring and the DCM-fermenting *Dehalobacter* strains. Primer/probe combinations were designed to quantify the CF-to-DCM-respiring and the DCM-fermenting *Dehalobacter* strains using qPCR (Table 3.1). The *Dehalobacter* sp. strain RM1 16S rRNA gene-targeted primer/probe set yielded the expected amplicons, and no amplification was observed when the cloned 16S rRNA genes of the *Dehalobacter* strain in culture Dhb-CF or *Dehalobacter restrictus* were used as DNA templates. Similarly, the primer/probe set designed for the quantification of the CF-to-DCM dechlorinating *Dehalobacter* strain in culture Dhb-CF did not generate fluorescence signals with template DNA of the DCM-degrading *Dehalobacter* sp. strain RM1; however, this primer/probe combination yielded amplicons when template DNA of the closely related *Dehalobacter restrictus* was used. qPCR targeting *Dehalobacter* 16S rRNA genes in genomic DNA extracted from non-augmented microcosm did not yield amplicons, indicating that CF-dechlorinating and DCM-fermenting *Dehalobacter* strains were absent in the site materials.

Table 3.1 Primer/probe sets used for 16S rRNA gene-targeted qPCR and qPCR standard curve information. Standard curves for quantifying the 16S rRNA gene copies of the CF-to-DCM-respiring *Dehalobacter* strain had a slope of -3.3, a y-intercept of 38, an R^2 value of 0.99, and an amplification efficiency of 99.3%. Standard curves for the *Dehalobacter* sp. strain RM1-specific primer and probe combination had a slope of -3.4, a y-intercept of 38, an R^2 value of 0.99, and an amplification efficiency of 96.9%.

Target	Primer/Probe Sequence
CF-respiring <i>Dehalobacter</i> sp.	Forward Primer 5'-CGACGCAACGCGAAGAA Reverse Primer 5'-CGAAGGGCACTCCCATATCTC Probe 5'-6-FAM-ACCAAGGCTTGACATCCAACCT-BHQ1
DCM-fermenting <i>Dehalobacter</i> sp. strain RM1	Forward Primer 5'-TCCCGCAACGAGCGCAACCCCTATA Reverse Primer 5'-TTTGTCAACGGCAGTCTATCCA Probe 5'-6-FAM-CAGTTACCAGCACGTAAAGGTGG-BHQ1

3.4.2 CF persistence in microcosms established with site materials and

groundwater. Microcosms established with site materials did not show increased CF removal relative to control microcosms, and the addition of electron donor did not stimulate CF degradation. Some CF loss (< 10 μ moles) occurred in all microcosms, most likely due to sorption to the solids and the stopper; however, ~70% of the initial dose of CF remained in the live and control microcosms after a 7-month incubation period. Microcosms established with site groundwater contained background aqueous phase CT concentrations of up to 35.5 μ M (5 mg L⁻¹). CF transformation products (i.e., DCM or CM) were not detected in any of the microcosms.

3.4.3 Fate of CF in microcosms augmented with the CF-to-DCM-dechlorinating

Dhb-CF culture. To explore if bioaugmentation affected CF fate, the CF-to-DCM-dechlorinating culture Dhb-CF was added to the microcosms. Somewhat unexpectedly, bioaugmentation of slurry microcosms prepared with un-purged site groundwater with culture Dhb-CF did not increase CF removal relative to heat-killed microcosms or non-

inoculated controls, and CF persisted even after an extended 7-month incubation period. In contrast, CF transformation was observed in microcosms established with phosphate-buffered mineral salts medium instead of site groundwater. CF was stoichiometrically transformed to DCM within 32 ± 11 days of augmentation in microcosms amended only with CF; a second CF addition was reductively dechlorinated to DCM within 6 ± 3 days (Figure 3.1A). Likewise, augmented microcosms that had been amended with both CF and DCM simultaneously produced stoichiometric amounts of DCM from CF (Figure 3.2A), but DCM was not transformed. Growth of the CF-to-DCM-dechlorinating *Dehalobacter* strain, as measured via 16S rRNA gene copy increase, occurred concomitant with CF-to-DCM transformation in all live incubations (Figures 3.1B and 3.2B). Live augmented microcosms showed a two orders of magnitude increase in *Dehalobacter* 16S rRNA gene copies from $4.67 \pm 2.63 \times 10^4$ to $1.48 \pm 1.06 \times 10^6 \text{ mL}^{-1}$, which equals an increase in total *Dehalobacter* cell numbers from $1.56 \pm 0.88 \times 10^6$ to $4.95 \pm 3.54 \times 10^7$. No CF dechlorination and no increase in 16S rRNA gene copies of the CF-to-DCM dechlorinating *Dehalobacter* sp. occurred in CF-amended microcosms that received a heat-killed inoculum or in non-inoculated controls (Figure 3.3A).

3.4.4 Successive bioaugmentation of CF-to-DCM-dechlorinating microcosms with the DCM-degrading consortium RM. After stoichiometric transformation of CF to DCM by culture Dhb-CF, microcosms were then augmented with the DCM-degrading consortium RM. Initially, no DCM degradation was observed in the microcosms augmented with consortium RM relative to control microcosms (Figures 3.1A and 3.2A). Some DCM loss ($< 9 \text{ } \mu\text{moles}$) occurred in all microcosms, most likely due to sorption; however, $\sim 78\%$ of the initial dose of DCM remained in control microcosms after a 7-month incubation period. After 105 days of incubation, 10 mM bicarbonate was added to

all microcosms (indicated by inverted triangles in Figures 3.1A and 3.2A), and DCM degradation started about 40 days following this addition. DCM was completely degraded within 106 ± 34 days of bicarbonate addition (i.e., 211 ± 34 days after bioaugmentation) in microcosms that had been initially amended with CF (Figure 3.1A). In microcosms that had received both CF and DCM, complete DCM degradation occurred approximately 217 ± 34 days after augmentation with consortium RM and approximately 112 days following bicarbonate addition (Figure 3.2A). Concurrent with DCM degradation, an increase in 16S rRNA gene copies of *Dehalobacter* sp. strain RM1 was observed (Figures 3.1C and 3.2C). 16S rRNA gene copies of the DCM-degrading *Dehalobacter* sp. strain RM1 were below the detection limit of about 3.00×10^2 copies mL^{-1} (i.e., 3 copies per qPCR reaction) following bioaugmentation and increased to $2.56 \pm 2.08 \times 10^4$ copies mL^{-1} (or $8.53 \pm 6.92 \times 10^5$ *Dehalobacter* sp. RM1 cells in the culture vessels, assuming three 16S rRNA gene copies per cell in the microcosms augmented with consortium RM). DCM degradation was strictly dependent on inoculation with consortium RM, and DCM persisted in all microcosms that did not receive this inoculum, including live microcosms amended with 10 mM bicarbonate (Figure 3.3B).

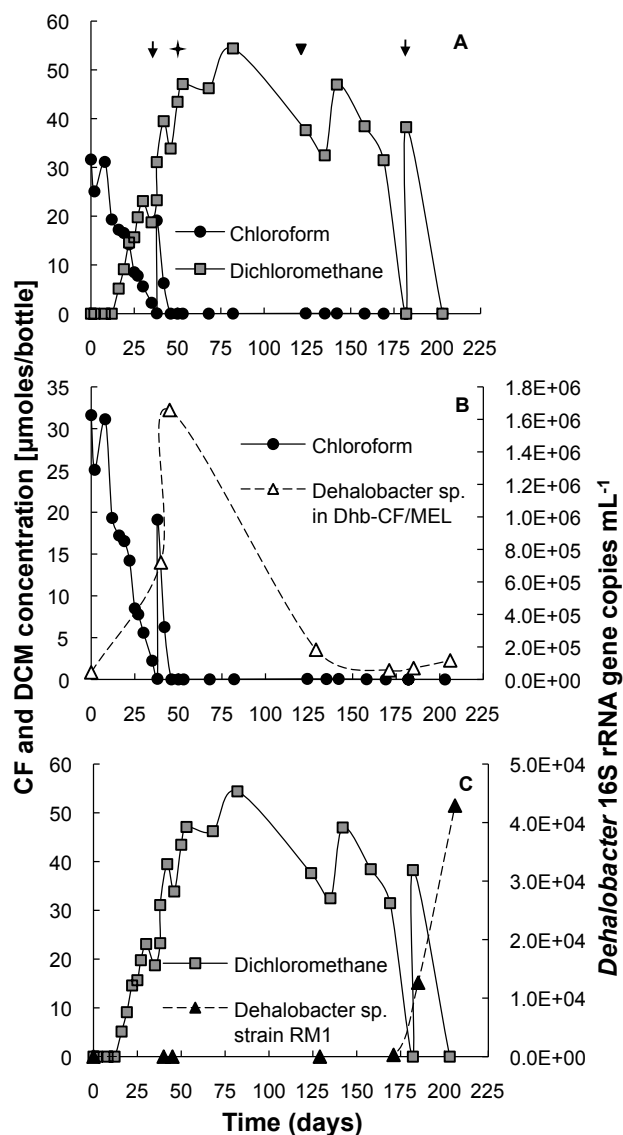


Figure 3.1 Sequential bioaugmentation with the Dhb-CF and the RM consortia in microcosms prepared with phosphate-buffered mineral salts medium and amended only with CF leads to complete detoxification. (A) CF (black circles) transformation by culture Dhb-CF (6% inoculum, v/v), followed by DCM (gray squares) degradation by consortium RM. Arrows indicate additional CF or DCM feedings. The star denotes augmentation with consortium RM (3% inoculum, v/v). The inverted triangle indicates the addition of 10 mM NaHCO_3 . (B) Increase in 16S rRNA gene copies of the CF-to-DCM-dechlorinating *Dehalobacter* strain in culture Dhb-CF (open triangles) concomitant with reductive dechlorination of CF. (C) Increase in the 16S rRNA gene copies of the DCM-dechlorofermenting *Dehalobacter* sp. strain RM1 in consortium RM (black triangles) concomitant with DCM degradation. The data shown are from one representative microcosm, and replicates performed similarly but exhibited somewhat variable lag times (± 11 days for CF; ± 34 days for DCM).

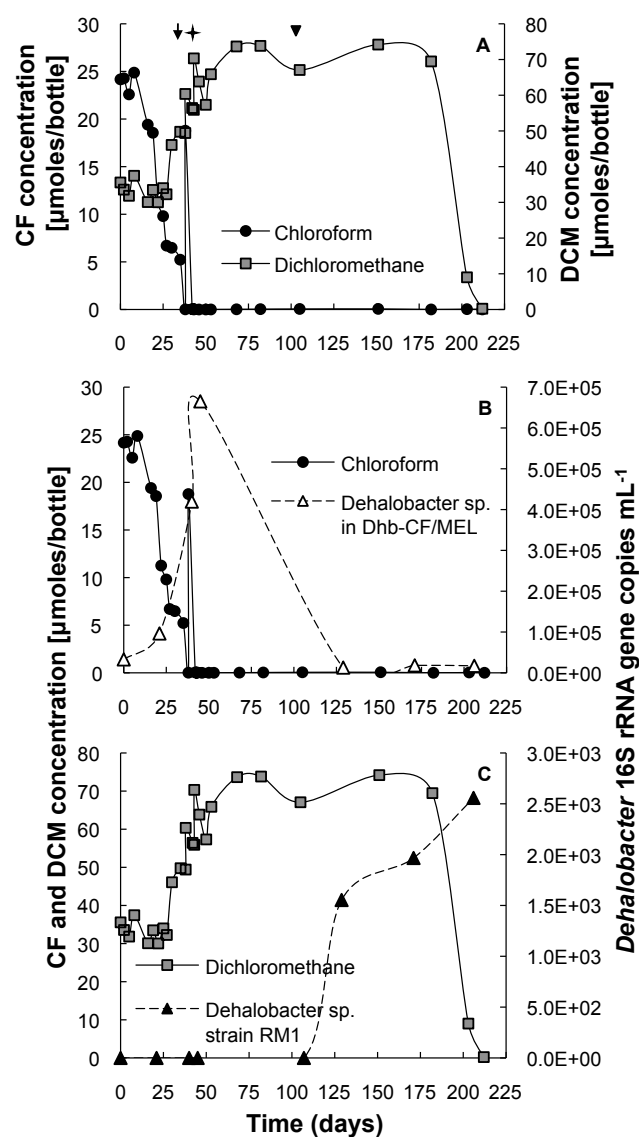


Figure 3.2 Sequential bioaugmentation with the Dhb-CF and the RM consortia in microcosms prepared with phosphate-buffered mineral salts medium and amended with CF and DCM leads to complete detoxification. (A) CF (black circles) transformation by culture Dhb-CF (6% inoculum, v/v), followed by DCM (gray squares) degradation by consortium RM. The arrow indicates an additional CF feeding. The star denotes augmentation with consortium RM (3% inoculum, v/v), and the inverted triangle indicates the addition of 10 mM NaHCO₃. (B) Increase in 16S rRNA gene copies of the *Dehalobacter* sp. in culture Dhb-CF (open triangles) concomitant with reductive dechlorination of CF. (C) Increase in the 16S rRNA gene copies of *Dehalobacter* sp. strain RM1 present in consortium RM (black triangles) concomitant with DCM degradation. The data shown are from one representative microcosm, and replicates performed similarly but exhibited somewhat variable lag times (± 2 days for CF; ± 38 days for DCM).

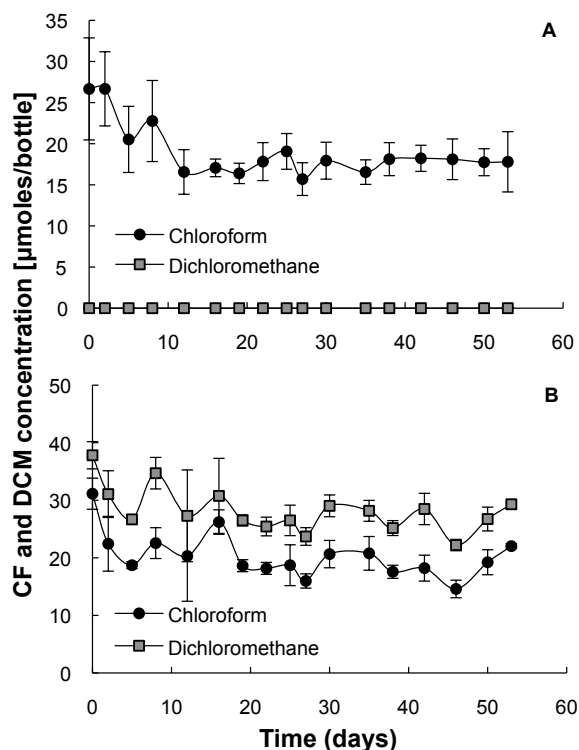


Figure 3.3 Lack of CF and DCM degradation in control microcosms. No CF (black circles) or DCM (gray squares) degradation was observed in microcosms augmented with autoclave-killed consortium Dhb-CF or in non-inoculated controls amended with (A) CF or (B) with CF and DCM. All data points represent average values from triplicate cultures, and the error bars represent one standard deviation.

3.4.5 Concomitant bioaugmentation with culture Dhb-CF and consortium RM. To

explore if the simultaneous augmentation with both culture Dhb-CF and consortium RM could initiate CF detoxification, duplicate microcosms were established. Concomitant augmentation of microcosms with culture Dhb-CF and consortium RM led to

stoichiometric reductive dechlorination of CF to DCM within 39 ± 5 days (Figure 3.4).

DCM degradation was not apparent initially, but started 5 days after a 10 mM

bicarbonate amendment. Quantification of the 16S rRNA gene copies mL^{-1} of the CF-to-DCM dechlorinating *Dehalobacter* strain increased from $1.90 \pm 0.84 \times 10^5$ to $1.82 \pm 1.51 \times 10^6$ during CF-to-DCM reductive dechlorination, and the 16S rRNA gene copies of the

DCM-degrading *Dehalobacter* strain increased from $4.95 \times \pm 0.91 \times 10^3$ to $1.27 \pm 0.77 \times 10^5$ copies mL⁻¹ during DCM degradation.

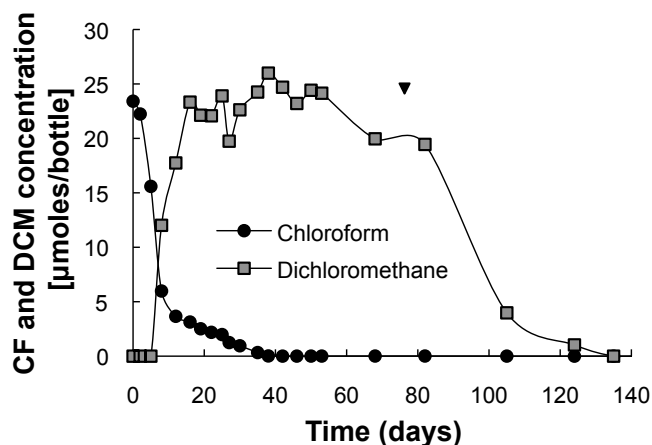


Figure 3.4 Concomitant bioaugmentation of microcosms with consortia Dhb-CF and RM leads to CF and DCM detoxification. CF (black circles) was stoichiometrically transformed to DCM (gray squares) by culture Dhb-CF. DCM was subsequently degraded by consortium RM following the addition of 10 mM NaHCO₃ (indicated by the inverted triangle). The data shown are from one representative microcosm, and replicates performed similarly but exhibited somewhat variable lag times (± 5 days for CF; ± 41 days for DCM).

3.5 Discussion

The discovery of *Dehalococcoides mccartyi* and the availability of PCE-to-ethene bioaugmentation consortia have provided a remedial alternative to achieve detoxification at sites impacted with chlorinated ethenes (14, 38, 41, 44, 49, 53). A similar approach to address chlorinated methane contamination is desirable because a large number of sites with CF as the primary contaminant exist, and innovative remedial approaches are needed. In addition, mixed contaminant plumes are common, and CF inhibits a variety

of microbial activities, including *Dehalococcoides* reductive dechlorination activity, thus limiting the success of microbial remedies at sites contaminated with both chlorinated ethenes and chlorinated methanes (5, 11, 21). The microbiology contributing to CF and DCM degradation has remained elusive but recent efforts identified distinct *Dehalobacter* populations involved in CF reductive dechlorination and DCM dechlorofermentation to innocuous products, highlighting the importance of this bacterial group for CF and DCM bioremediation (23, 31, 35). The goal of the current study was to explore the feasibility of bioaugmentation for achieving CF and DCM detoxification.

Both CF and DCM persisted at the study site and in microcosms prior to bioaugmentation indicating that the indigenous microbes did not have the capacity to efficiently degrade CF and DCM. qPCR data supported this assessment, and no *Dehalobacter* 16S rRNA gene sequences were detected. The lack of indigenous degradation activity may also be due to unfavorable conditions. For example, electron donor availability often limits reductive dechlorination activity; however, zero-valent iron PRBs generate hydrogen, an electron donor supporting organohalide-respiring *Dehalobacter* populations (23, 25, 30, 47). Hence, the persistence of CF *in situ* was likely not due to electron donor limitation. An alternate explanation is CT toxicity, which has been reported to inhibit microbial activity (5, 9). Although the PRB significantly reduced the CT concentrations, up to 5 mg L⁻¹ CT were measured in the microcosms established with groundwater. No CT was detected in microcosms prepared with phosphate-buffered mineral salts medium indicating that CT was mainly associated with the groundwater and not with the solids. Accordingly, bioaugmentation achieved CF detoxification in microcosms established with medium but not in the microcosms established with site groundwater. Both, the CF-to-DCM-dechlorinating *Dehalobacter* strain present in culture Dhb-CF and the DCM-

degrading *Dehalobacter* sp. strain RM1 present in consortium RM were sensitive to CT. Apparently, the amount of CT was sufficient to inhibit CF dechlorination and DCM degradation by consortia Dhb-CF and RM. The CT sensitivity of both the CF-respiring and the DCM-fermenting *Dehalobacter* strains indicates that CT removal is a prerequisite for the implementation of anaerobic biological remedies that lead to detoxification at CF- and DCM-impacted sites. Although mechanistic understanding of how microbial populations can contribute to CT removal is developing, microbes that can use CT as an energy source (e.g., via organohalide respiration) have not been found (20). CT reductive dechlorination is energetically favorable (i.e., could theoretically support energy conservation via organohalide respiration); however, the toxicity of the trichloromethyl radical formed during fortuitous reactions with certain redox active molecules produced by many microbes may present an insurmountable barrier for organohalide-respiring bacteria (18, 46, 52). Nevertheless, a successful field bioaugmentation demonstration with *Pseudomonas stutzeri* strain KC achieved CT degradation *in situ*, demonstrating that microbial remedies are feasible if mechanistic understanding of the degradation process has been developed and the site is carefully managed (12, 13).

CF dechlorination rates in microcosms prepared with phosphate-buffered mineral salts medium and augmented with culture Dhb-CF increased following repeated CF additions, indicating that the CF-respiring strain introduced with the inoculum grew, a finding supported by the qPCR results. CF-to-DCM dechlorination activity occurred in the presence of DCM suggesting that CF reductive dechlorination is feasible at sites where DCM is present, as long as DCM concentrations do not exceed 200 mg L⁻¹ (~2.5 mM), the reported inhibitory concentration for the CF-respiring strain in culture Dhb-CF (23).

The DCM produced through CF dechlorination by culture Dhb-CF was fermented in microcosms augmented with consortium RM. DCM degradation rates in microcosms augmented with consortium RM also increased following repeated DCM additions indicating that strain RM1 grew via the utilization of DCM, a finding supported by the qPCR results. CF-to-DCM dechlorination sustained growth of an organohalide-respiring *Dehalobacter* strain in culture Dhb-CF to a yield of $3.58 \pm 2.58 \times 10^6$ 16S rRNA gene copies per $\mu\text{mol Cl}^-$ released. DCM fermentation supported growth of *Dehalobacter* sp. strain RM1, and $1.80 \pm 1.48 \times 10^4$ 16S rRNA gene copies per $\mu\text{mol Cl}^-$ released were calculated. The growth yields for both the organohalide-respiring and the DCM-dechlorofermenting *Dehalobacter* strains in the microcosm experiments were about one order of magnitude lower than those previously reported in enrichment cultures without aquifer solids (23, 31). This is a relevant observation because growth yields determined in sediment-free mixed and pure cultures under laboratory conditions are generally used for *in situ* biomass yield and rate predictions. The reasons why growth yields per mole of chlorinated substrate consumed differ for the same organism in microcosms containing solids versus solids-free enrichment cultures are unclear but warrant further exploration. We considered differences in DNA extraction efficiencies to explain this discrepancy, but determined this not to be a source of error because the procedure applied extracted similar amounts of DNA from samples with or without solids. Further, similar 16S rRNA gene copy numbers were calculated from qPCR data generated with undiluted and 1:10 diluted template DNA samples, indicating that PCR inhibition did not occur. Also noticeable is the two order of magnitude difference in growth yields between the organohalide-respiring *Dehalobacter* strain in culture Dhb-CF and the DCM-fermenting *Dehalobacter* strain in consortium RM. Both CF reductive dechlorination to DCM and DCM dechlorofermentation are associated with a considerable change in free

energy, and -170.8 kJ for CF-to-DCM reductive dechlorination and -215.56 kJ for DCM fermentation to acetate are available under standard conditions (10, 19). Theoretically, DCM fermentation to acetate yields more energy than CF reductive dechlorination to DCM; however, it appears that not all of this available free energy is conserved given the significantly lower growth yield attained by the DCM-fermenting *Dehalobacter* sp. strain RM1. The disparity amid energy production and growth yield for DCM fermentation by strain RM1 may perhaps be explained by an unaccounted burden to the cell during DCM fermentation. Another possible explanation is that multiple organisms are involved in the overall fermentation of DCM to acetate in consortium RM. Syntrophic associations between *Dehalobacter* sp. strain RM1 and other members of consortium RM (e.g., acetogenic bacteria) would result in an apportionment of the energy produced and, consequently, lower growth yields for the individual populations involved. Despite the low growth yield of the DCM-fermenting *Dehalobacter* sp. strain RM1, it is important to emphasize that a relatively small inoculum size achieving initial cell titers of 10^2 - 10^3 cells mL^{-1} of microcosm suspension was sufficient to result in complete DCM degradation. Even after repeated DCM feedings, the strain RM1 numbers did not exceed 1.8×10^5 16S rRNA gene copies mL^{-1} .

The DCM-degrading consortium RM requires bicarbonate to efficiently degrade DCM (31). The microcosms established in mineral salts medium used phosphate buffer without bicarbonate additions because we assumed that the bicarbonate associated with the solids and the CO_2 produced during lactate fermentation would fulfill the requirements of the DCM-fermenting *Dehalobacter* sp. strain RM1. DCM degradation only started after amending the microcosms with NaHCO_3 (Figures 3.1A, 3.2A and 3.4) suggesting that the indigenous amounts of bicarbonate/ CO_2 were insufficient. Notably,

the addition of 10 mM bicarbonate did not alter the pH of the microcosms, which remained circumneutral for the duration of the experiment. The requirement for bicarbonate is not unprecedented, and *Dehalobacterium formicoaceticum* – the only other anaerobe capable of DCM degradation described to date – required bicarbonate in order to ferment DCM to acetate, formate and chloride (42). Apparently, *Dehalobacter* sp. strain RM1 in consortium RM and *Dehalobacterium formicoaceticum* share the requirement for bicarbonate. Therefore, it is possible that shorter lag times prior to DCM degradation would have been observed if the microcosms were amended with bicarbonate at the onset of the experiments. Groundwater bicarbonate concentrations vary greatly, but generally exceed 10 mg L⁻¹ (0.16 mM) (29). CO₂-releasing processes, such as microbial catabolism of reduced carbon substrates and the metamorphosis of carbonaceous rocks, occur in groundwater reservoirs and can lead to bicarbonate concentrations that surpass 1,000 mg L⁻¹ (16 mM), a concentration well above that amended to the augmented microcosms (29). The minimum available bicarbonate concentration to sustain DCM fermentation is not known; however, the addition of bicarbonate solutions to aquifers for the purpose of pH adjustment is a common practice suggesting that biostimulation of DCM fermentation with bicarbonate additions is feasible at field sites with low bicarbonate groundwater.

Another issue that affects DCM degradation is the presence of CF. A previous study reported inhibition of DCM degradation by consortium RM in the presence of CF concentrations as low as 5 mg L⁻¹ (31). Hence, the initial concentration of 25 mg CF L⁻¹ could explain the long lag time before DCM degradation activity occurred in the microcosms that received both inocula simultaneously (Figure 3.4). Apparently, the DCM-degrading *Dehalobacter* sp. strain RM1 was maintained during this phase of

inhibitory CF concentrations and lack of bicarbonate, and grew with DCM after CF concentrations declined and bicarbonate was added.

CF was completely detoxified in microcosms that received both consortia together at the start of the experiment or sequentially (i.e., consortium RM was added after CF had been dechlorinated to DCM). These results suggest that an augmentation approach that combines culture Dhb-CF and consortium RM is effective to remediate CF-contaminated sediments. Combined consortia established by physically blending separate enrichment cultures with distinct degradation capabilities have been shown to be effective previously. For example, culture MS, which reductively dechlorinates 1,1,1-trichloroethane (1,1,1-TCA) to 1,1-dichloroethane and chloroethane, was used to relieve the 1,1,1-TCA-mediated inhibition of TCE dechlorination by consortium KB-1 (24). Bioaugmentation of microcosms containing 1,1,1-TCA and TCE with a mixture of consortia MS and KB-1 effectively transformed 1,1,1-TCA and TCE to chloroethane and ethene. These observations suggest that blending consortia such as Dhb-CF with RM can be productive and offer remedial strategies at sites that harbor contaminant mixtures. Current efforts explore if a Dhb-CF/RM blend maintains its ability to reductively dechlorinate CF to DCM and dechloroferment DCM to innocuous products. Future pilot test studies at CF-contaminated sites are needed to evaluate if a Dhb-CF/RM blend or subsequent inoculation (i.e., augmentation with Dhb-CF followed by augmentation with RM) will achieve efficient *in situ* detoxification of CF and/or DCM in anoxic aquifers.

Monitoring of the bacterial populations involved in contaminant detoxification is a critical component of successful bioremediation (39). qPCR targeting gene(s) or transcripts of

interest has emerged as a powerful tool for site assessment and linking the presence, abundance and activity of the organism of interest with the observed contaminant transformation processes (36). To monitor the CF-respiring and the DCM-fermenting *Dehalobacter* strains individually, specific qPCR primer/probe sets were designed to target the 16S rRNA gene sequence of each *Dehalobacter* strain. Testing of these primer/probe sets demonstrated that the assays achieved the intended specificity. Since *Dehalobacter* 16S rRNA gene sequences were not detected in materials from the study site prior to bioaugmentation (i.e., neither primer set yielded amplicons), native *Dehalobacter* populations did not interfere with the monitoring of the CF-dechlorinating and DCM-fermenting *Dehalobacter* strains. The *Dehalobacter* clade has few cultured representatives and the phylogenetic diversity of *Dehalobacter* 16S rRNA gene sequences is currently unclear. Consequently, the specificity of *Dehalobacter* 16S rRNA gene-targeted primers must be carefully evaluated to ensure that only the genes of the target strains are amplified. This seems particularly important when the qPCR approach is applied to sites that contain native *Dehalobacter* populations. The *Dehalobacter* 16S rRNA gene-specific qPCR assays used in this study distinguished the CF-to-DCM-dechlorinating and the DCM-fermenting *Dehalobacter* strains and were valuable for monitoring the different *Dehalobacter* populations in the microcosms. Because the *Dehalobacter* 16S rRNA gene diversity in aquifers is poorly understood, the general applicability of these primer/probe sets for site assessment and bioremediation monitoring is currently unclear. Verification with a larger set of environmental samples from a variety of CF- and DCM-contaminated sites is needed to explore if robust links between specific *Dehalobacter* 16S rRNA genes and distinct dechlorination/degradation activities can be established.

3.6 Acknowledgments

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CHAPTER 4

FRACTIONATION OF STABLE CARBON ISOTOPES DURING DEGRADATION OF DICHLOROMETHANE BY THE *DEHALOBACTER* SP. STRAIN RM1-CONTAINING CONSORTIUM RM

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4.1 Introduction

The chlorinated methanes – carbon tetrachloride (CT), chloroform (CF), dichloromethane (DCM) and chloromethane (CM) – have been commonly used as organic solvents for degreasing in dry cleaning, electronics, industrial manufacturing and machine maintenance industries since the 1960s (2-4, 29-32). In addition, these chlorinated aliphatic hydrocarbons currently serve as feedstock materials for the manufacturing of silicones and chlorofluorocarbons, among other widely used chemicals (2-4). The extensive use of chlorinated methanes, as well as improper handling and storage, has released significant amounts of these chemicals into the environment. As a result, CT, CF and DCM are increasingly been detected in soil and groundwater. DCM, in particular, has been detected in at least 882 of 1,569 National Priority List (NPL) sites identified by the U.S. Environmental Protection Agency (EPA), making it the sixth most frequently detected organic contaminant in groundwater from hazardous waste sites (1, 7). The U.S. EPA considers DCM to be a probable human carcinogen and has classified DCM as a priority contaminant with a maximum contaminant level (MCL) in drinking water of 0.005 mg DCM L⁻¹ (9, 10).

Although persistent at many contaminated sites, DCM can also be subject to biodegradation under both aerobic and anaerobic conditions. Aerobically, DCM can be degraded to formaldehyde and inorganic chloride by methylotrophic bacteria containing glutathione-dependent DCM dehalogenases (6, 19-21, 26, 36). Under anaerobic conditions, the homoacetogenic bacterium *Dehalobacterium formicoaceticum* has been shown to ferment DCM to acetate, formate and inorganic chloride (24, 25). Recently, two anaerobic *Dehalobacter*-containing consortia were described to share the same DCM dechlorofermenting capabilities as *Dehalobacterium formicoaceticum* – an enrichment culture derived from subsurface soil collected from an aquifer in Sydney, Australia with an extensive history of contamination with chlorinated solvents, and consortium RM (18, 22). Consortium RM was derived from pristine river sediment and contains *Dehalobacter* sp. strain RM1, which grows concomitant with DCM fermentation (18). Laboratory-scale treatability studies using microcosms established with aquifer material from a contaminated industrial site impacted with CF revealed that bioaugmentation with consortium Dhb-CF – a CF-to-DCM dechlorinating consortium that also contains a distinct *Dehalobacter* strain – and consortium RM resulted in the complete detoxification of CF and DCM (15)(Justicia-Leon et al. 2012, submitted). These results suggest that bioaugmentation with consortium RM might prove to be effective as an *in situ* bioremediation treatment at sites contaminated with DCM.

The application of bioremediation for contaminant detoxification *in situ* has its challenges. One of the principal difficulties that bioremediation practitioners confront is providing evidence that bioremediation is occurring, especially when chemical measurements offer insufficient data. In addition, the provided evidence must confirm that a decrease in contaminant concentration is a direct result of the remedial action

employed, and not due to physical processes such as evaporation, dilution or sorption to aquifer material. The use of compound specific isotope analysis (CSIA) as a means to provide such evidence has increased in the past two decades (8, 27, 35).

CSIA allows for the measurement of stable isotope ratios at the elemental level in environmental samples. Every element that comprises the molecules of a given compound has a characteristic isotopic signature (i.e., relative abundance of the heavy and light isotopes of each element within the compound) (8). Although the isotopes of an element can be nearly identical, the chemical bonds formed by a lighter isotope are weaker than the bonds involving the heavier isotope. This difference in bond strength results in a higher reactivity for the bonds formed by the lighter isotope, and thus, preferential removal of these lighter isotopes from the compound, leading to isotopic fractionation during the degradation process (27). The relative abundance of the heavy (hE) and light (lE) isotopes of a given element (E) within a particular compound in a sample can be determined by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) and are expressed as a ratio (R) where $R = ^hE/^lE$ (8). Conventionally, these ratios are averaged over the bulk compound to calculate bulk isotope ratios, which are stated as difference in per mil (δ^hE) with respect to an internationally accepted reference standard, as given by the following equation: $\delta^hE = [(R - R_{ref})/R] \times 1000\text{‰}$ (8). By exploring the changes in these ratios during the course of biodegradation (or other processes that lead to a measurable decrease in contaminant concentration), CSIA can provide an insight into the fate of target contaminants (17). Notably, bulk isotopic ratios are a function of the degradation pathway, thus, CSIA can potentially distinguish biodegradation from other physical or chemical processes that can also lead to contaminant degradation. CSIA has been successfully implemented to evaluate and

monitor bioremediation of contaminants in both laboratory-scale treatability studies and *in situ* (12, 14, 27, 33, 35, 39). In particular, two previous studies have explored the stable carbon isotope fractionation during the degradation of DCM under aerobic and anaerobic (denitrifying) conditions (16, 34). The results of these studies demonstrate a significant stable carbon isotope fractionation during DCM degradation for the ten bacterial strains tested, but suggest no consistent differences in fractionation for the two conditions examined (i.e., aerobic vs denitrifying) (34). Notably, in all of the bacterial strains previously tested, DCM degradation is carried out by glutathione-dependent dehalogenases involved in nucleophilic substitution reactions (16, 34).

To explore the feasibility of applying CSIA to monitor the degradation of DCM by consortium RM under fermentative conditions, microbial culture experiments were performed to assess the fractionation of stable carbon isotopes during the fermentation of DCM by *Dehalobacter* sp. strain RM1.

4.2 Materials and Methods

4.2.1 Consortium RM maintenance and medium preparation. Consortium RM was derived from sediments collected from Rio Mameyes in Puerto Rico (18). Cultures were inoculated (3% vol/vol) into defined, anoxic, reduced mineral salts medium containing 30 mM sodium bicarbonate (NaHCO_3) and 10 mM HEPES (pH 7.2) in vessels containing a N_2/CO_2 (80/20, vol/vol) mixture as headspace (23). Consortium RM vessels were amended with DCM ($\sim 140 \text{ mg L}^{-1}$, aqueous phase concentration). Two sets of triplicate vessels, one set consisting of 160-mL glass bottles (nominal capacity) and another consisting of 250-mL glass bottles (nominal capacity), were incubated statically at room

temperature. Triplicate heat-killed (i.e., autoclaved) and abiotic (no inocula) incubations served as negative controls.

4.2.2 Analytical methods. DCM was monitored via manual headspace injections (100 μL) into a Hewlett-Packard (HP) 6890 gas chromatograph (GC) equipped with an electron capture detector (ECD) as described (18). The method provided a linear detector response for DCM concentrations from 1.5 μM to 385 μM . Standards were prepared by adding known amounts of DCM to culture vessels containing medium and aqueous phase concentrations were calculated using 0.0895 as the dimensionless Henry's law constant (13).

4.2.3 Stable isotope analysis. Compound-specific isotope analysis of DCM was performed using gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) on an Agilent 6890 GC system connected to a Finnigan MAT Delta+-XL IRMS. The GC column used was a Supelco VOCOL fused capillary column (60 m x 0.32 mm; film thickness, 3 μm internal diameter). For sample separation and elution, the GC oven temperature was initially held at 60°C for 1 minute, then increased at 5°C min^{-1} up to 110°C, and held isothermally at 110°C for 2 minutes. Since sample aliquots contained no headspace, a 5-mL gastight syringe with Mininert valve attachment was used to transfer 2 mL of sample into a 4-mL (nominal capacity) borosilicate vial, which was then capped and wrapped with paraffin film. The original sample aliquots, now with a 2-mL headspace were then allowed to equilibrate overnight. Samples with DCM concentrations greater than 50 mg L^{-1} (aqueous phase concentration) were analyzed by headspace injection. Headspace gas samples were injected into a 180°C injector port using a 1-mL gastight syringe at an inlet split ratio setting of 6:1. Samples with DCM

concentrations below 50 mg L⁻¹ were analyzed by purge and trap (Teledyne Tekmar Velocity XPT Purge and Trap Sample Concentrator) integrated via an Agilent volatiles interface with the GC/C/IRMS configuration mentioned above. Liquid samples were taken using a 5-mL gastight syringe with a Mininert valve adapter and injected into the purge and trap sample concentrator through a 20- μ m filter attachment. Samples were flushed with a 40 mL min⁻¹ stream of helium for 11 minutes and trapped onto a VOCARB 3000 trap (Supelco). Samples were then desorbed at 250°C for 2 minutes at 50 mL min⁻¹ helium flow rate. Subsequently, the trap was baked for 10 minutes at 255°C. Samples entered the GC/C/IRMS configuration mentioned above at an inlet split ratio of 15:1 or 40:1 depending on sample concentration. Isotopically characterized DCM aqueous controls, analyzed by headspace and purge and trap, were within error of the known values for the $\delta^{13}\text{C}$ standard (- 39.8‰), and had a standard deviation of < 0.3‰ (n = 53). However, total analytical uncertainty for each sample is reported as $\pm 0.5\%$ to incorporate both accuracy and reproducibility as described by Sherwood Lollar et al. (37).

4.3 Results and Discussion

4.3.1 Enrichment of ^{13}C during DCM degradation by consortium RM. In treatment vessels amended with DCM and inoculated with consortium RM, DCM degradation began after a lag period of 12 days. DCM was completely degraded within 10 days after the lag period in vessels containing 200 mL total liquid volume (250-mL glass vessels, nominal capacity) and within 16 days after the lag period in vessels containing 130 mL total liquid volume (160-mL glass vessels, nominal capacity) (Figure 4.1). Once DCM

degradation started, the DCM degradation rates for consortium RM were 39.26 ± 4.49 and 14.90 ± 3.95 $\mu\text{moles DCM day}^{-1}$ in 160-mL and 250-mL vessels, respectively. In all live incubations, DCM became significantly enriched in ^{13}C during the degradation process. $\delta^{13}\text{C}$ isotope values for DCM during biodegradation by consortium RM ranged from - 40.0‰ at inoculation, to + 43.0‰ at the conclusion of the analysis. Heat-killed and abiotic controls showed no enrichment in ^{13}C over an incubation period of 28 days ($\delta^{13}\text{C} = - 39.9 \pm 0.2$ ‰) (Figure 4.2).

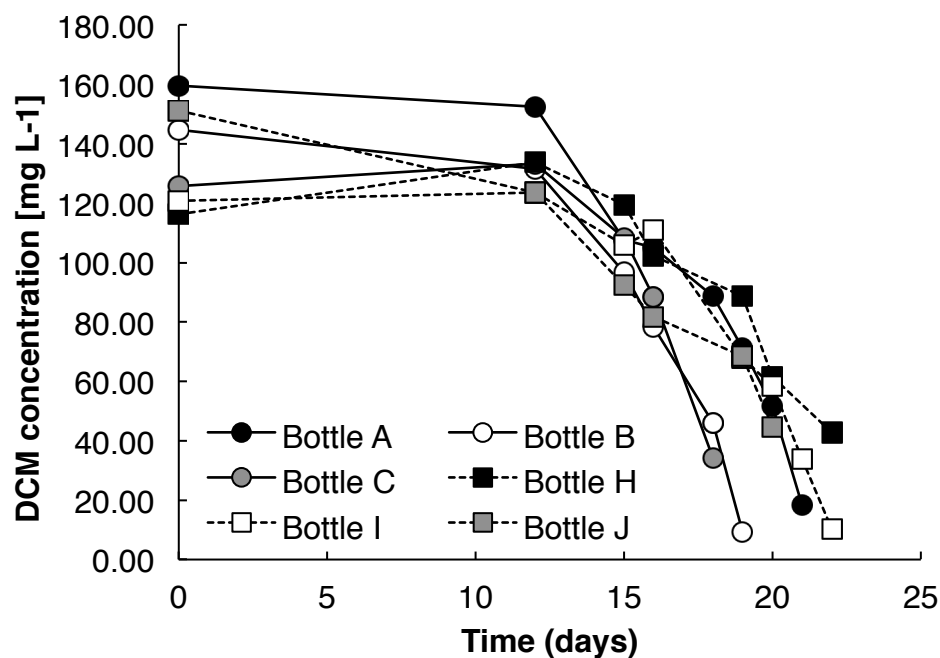


Figure 4.1 DCM concentration (mg L^{-1}) decreased with incubation time (days) in inoculated vessels. Solid black lines represent culture vessels containing 200 mL medium in 250-mL (nominal capacity) serum bottles. Dotted black lines represent culture vessels containing 130 mL medium in 160-mL (nominal capacity) serum bottles.

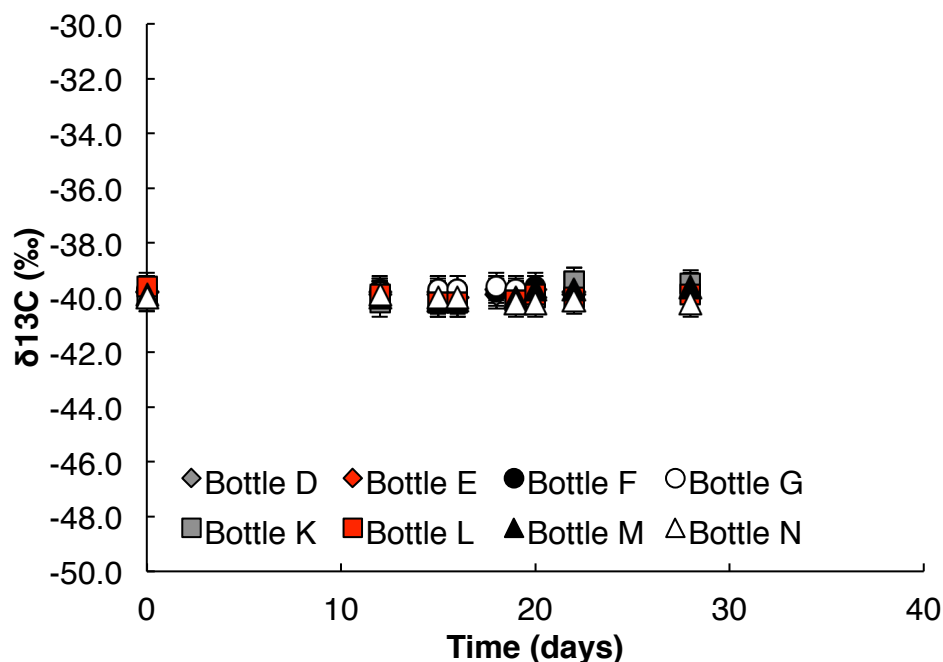


Figure 4.2 $\delta^{13}\text{C}$ (‰) vs. time (days) for heat-killed controls (bottles F, G, M, N) and abiotic controls (bottles D, E, K, L). All controls were identical to the known $\delta^{13}\text{C}$ of the DCM standard within $\pm 0.5\text{‰}$.

4.3.2 Bulk stable carbon isotopes enrichment factors (ϵ_{bulk}). The Rayleigh model described the isotope fractionation during degradation of DCM by consortium RM in all live incubations. For each of the six experimental vessels (3 160-mL vessels and 3 250-mL vessels), Rayleigh models were generated and demonstrated R^2 values ranging from 0.84 to 0.99. The bulk stable carbon isotopes enrichment factor (ϵ_{bulk}) for each experimental replicate vessel, however, did not always lie within the 95% confidence interval range (Table 4.1a). In accordance with previous reports, data points with a low fraction of DCM remaining (i.e., less than 10% of the initial DCM concentration, $f < 10\%$) were omitted to avoid high uncertainty in quantification, as well as the significant propagated error that arises from multiple vessel samplings (5, 28). In addition, vessel H was omitted from Rayleigh calculations because the initial DCM concentration was lower

than subsequent concentration measurements following the start of biodegradation (Figure 4.1).

Rayleigh models were recalculated on data subsets for treatments A, B, C, I and J omitting data points with $f < 10\%$ (Table 4.1b). As a result, the variability of ϵ_{bulk} values was greatly reduced. In fact, since all ϵ_{bulk} values were within the 95% confidence interval of each other, one Rayleigh model was calculated using the cumulative data for all five bottles (Table 1b). The combined Rayleigh ϵ_{bulk} value of bottles A, B, C, I and J ($f > 10\%$) was $-23.8 \pm 2.2\%$ (Figure 4.3).

Table 4.1 The Rayleigh models calculated for each inoculated experimental vessels including all data points (A) and omitting data points with a DCM fraction remaining (f) below 10% (B). Bolded characters denote Rayleigh models that changed following omission of points ($f < 10\%$).

	Bottle	Epsilon (ϵ)	Alpha (α)	r^2	95% CI (2σ)
(A)	A	-25.0	0.9750	0.98	± 3.5
	B	-29.7	0.9703	0.98	± 4.8
	<i>All Points</i> C	-25.0	0.9750	0.99	± 3.7
	H	-17.1	0.9829	0.89	± 6.1
	I	-31.5	0.9685	0.96	± 5.6
	J	-24.7	0.9753	0.84	± 12.3
(B)	A	-25.0	0.9750	0.98	± 3.5
	B	-24.3	0.9757	0.95	± 8.1
	C	-25.0	0.9750	0.99	± 3.7
	<i>$f > 10\%$</i> I	-22.1	0.9779	0.97	± 3.7
	J	-24.7	0.9753	0.84	± 12.3
	A, B, C, I, J	-23.8	0.9763	0.93	± 2.2

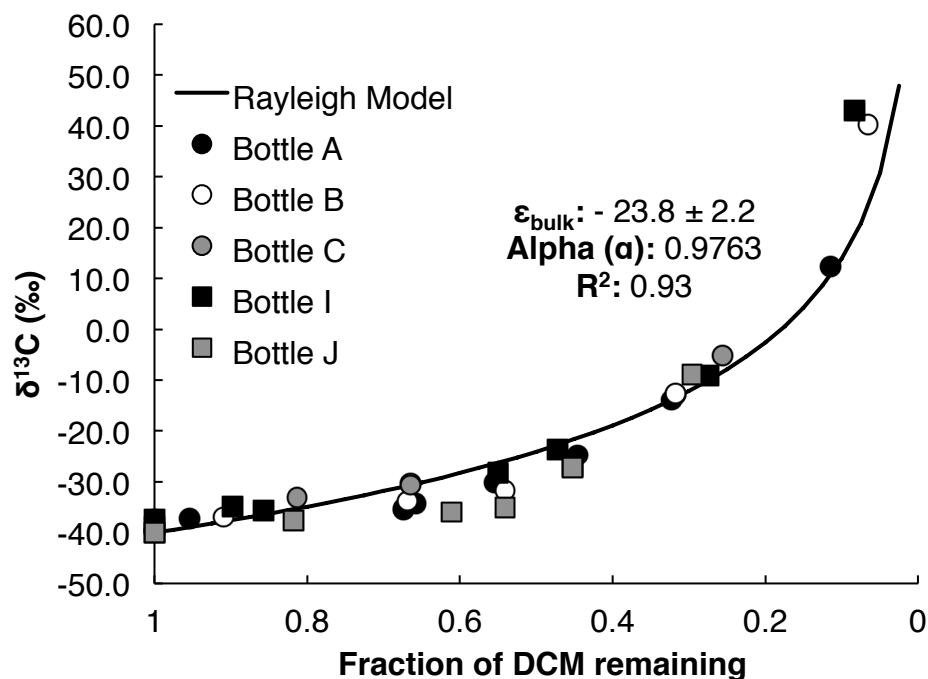


Figure 4.3 Rayleigh model for all experimental vessels inoculated with consortium RM. The combined Rayleigh ϵ_{bulk} value of bottles A, B, C, I and J ($f > 10\%$) was $-23.8 \pm 2.2\text{‰}$.

4.3.3 Kinetic isotope effect (KIE) values. Kinetic isotope effect (KIE) values describe the preferential chemical bond breakage of the lighter to heavier carbon isotopes ($^{12}\text{C}/^{13}\text{C}$). KIEs can be linked to the type of chemical bonds broken (e.g., C-Cl), the number of bonds broken, and the enzymatic kinetics of a reaction pathway. KIEs are inversely related to the fractionation factor (α) derived from the Rayleigh model; hence, CSIA can possibly provide insights into the reaction kinetics for biotic DCM degradation.

In previous studies, bacteria that degrade DCM via thiolytic dehalogenation under denitrifying conditions have been found to have KIE values ranging from 1.048 to 1.065 (34). Thiolytic dehalogenation is the particular pathway that methylotrophic bacteria

employ to catalyze DCM degradation via glutathione S-transferases to produce chloride and S-chloromethylglutathione (11). Upon formation, S-chloromethylglutathione is further hydrolyzed to glutathione, chloride, and formaldehyde (11). In contrast to previously published KIEs, the KIE value for DCM fermentation by *Dehalobacter* sp. strain RM1 in consortium RM was experimentally estimated to be 1.024. The difference between the KIEs estimated for *Dehalobacter* sp. strain RM1 (1.024) and for methylotrophic bacterial strains (1.048 to 1.065) suggests that there must be a corresponding difference in DCM biodegradation pathways. One possible explanation for the variation among KIEs could be a dissimilarity in the symmetry of the transition states formed as DCM is degraded. As transition states become more symmetrical, reactions tend to follow the S_N2 -type mechanism yielding a transition state in which two molecules are involved (38). Conversely, S_N1 reactions, which have a lower transition state symmetry, tend to have smaller KIE values. Smaller KIEs in S_N1 reactions are due to increased bonding to stabilize the central carbon atom as a result of the loss of the leaving group (38). Elsner et al. reported a similar trend with experimentally determined KIEs for various S_N1 -type (1.00-1.03) and S_N2 -type (1.03-1.09) reactions (8).

The glutathione-dependent dehalogenation pathway has two transition states, both exhibiting a bimolecular nucleophilic substitution (S_N2). Based on expected KIE values reported by Elsner et al., it is unlikely that the DCM degraded by *Dehalobacter* sp. strain RM1 is undergoing dechlorination via the thiolytic dehalogenation pathway (8). One possible pathway to explore would be the fermentation pathway suggested by Mägli et al. for *Dehalobacterium formicoaceticum* (24). Mägli and colleagues propose that, in cultures of *Dehalobacterium formicoaceticum*, DCM and tetrahydrofolate are converted by one or more enzymatic reactions to inorganic chloride and methylene

tetrahydrofolate. The intermediates of the proposed fermentation reaction remain unknown, impeding correlations to be made between the DCM fermentation pathway thought to be followed by *Dehalobacterium formicoaceticum* and that of *Dehalobacter* sp. strain RM1. Nevertheless, based on the difference in KIE values between S_N1 - and S_N2 -type reactions, we can hypothesize that the DCM being degraded by *Dehalobacter* sp. strain RM1 in consortium RM is likely undergoing an S_N1 -type nucleophilic transition state, with an associated smaller KIE value.

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CHAPTER 5

MICROBIAL COMMUNITY CHANGES DURING ENRICHMENT WITH DICHLOROMETHANE AS THE SOLE ENERGY SOURCE

Reproduced in part with permission from Justicia-Leon, S. D.; Simsir, B.; DeLeon-Rodriguez, N.; Konstantinidis, K. T.; Löffler, F. E. Microbial community changes during enrichment with dichloromethane and identification of a dichloromethane-degrading *Dehalobacter* sp. in a pristine river sediment. In preparation. All copyright interests will be exclusively transferred to the publisher upon submission.

5.1 Introduction

Dichloromethane (DCM), also known as methylene chloride, is a halogenated aliphatic hydrocarbon with widespread applications as a solvent in many chemical and manufacturing processes. Since its first preparation in the mid 1840s, DCM has been used as a solvent in the pharmaceutical, electronics, manufacturing, and paint and coating industries, as well as in some agricultural applications (1, 2, 36). The widespread use of DCM and improper handling and disposal practices have caused DCM to become a common groundwater contaminant. Recent estimates of global DCM emissions report, on average, 560,000 metric tonnes DCM are released into the environment every year as a consequence of industrial practices (15, 33). Contaminant surveys performed by the U.S. Environmental Protection Agency (EPA) have identified DCM in approximately 56% of all National Priority List (NPL) sites across the country. As a result of the extensive contamination, the toxicity of DCM, and the potential for human exposure, DCM has been included on the U.S. Agency for Toxic Substances and Disease Registry's (ATSDR) Substance Priority List (SPL) of hazardous contaminants (18).

Interestingly, DCM is also produced from natural sources. Oceans and biomass burning account for approximately 190,000 and 60,000 metric tonnes DCM released yr^{-1} (25, 28). In addition, several species of phytoplankton (i.e., *Rhodomonas salina*, *Karenia brevis*, *Pleurochrysis carterae* and *Chaetoceros neogracilis*) have been shown to produce significant amounts of DCM (13, 39). Evidence of natural production of DCM suggests that DCM is not a purely synthetic compound, and microorganisms have been exposed to this compound long before the anthropogenic production began. Hence, it is likely that microbes have developed mechanisms to utilize DCM as a source for carbon and/or energy.

Several DCM-utilizing microorganisms have been isolated and/or identified to date. Under oxic conditions, DCM is readily degraded by methylotrophs, and at least seven aerobic methylotrophic bacterial species have been shown to dehalogenate DCM to formaldehyde and inorganic chloride via reactions involving cytosolic glutathione transferases(45). Far less is known about the degradation of DCM under anoxic conditions. Only *Dehalobacterium formicoaceticum* and two *Dehalobacter*-containing consortia have been described to perform DCM degradation in the absence of oxygen (24, 27, 30, 31). *Dehalobacterium formicoaceticum* and consortium RM ferment DCM to organic acids (i.e., acetate, formate) and inorganic chloride (24, 30). Furthermore, *Dehalobacter* sp. strain RM1 in consortium RM was shown to grow concomitant with DCM fermentation (24).

Bacteria utilizing DCM under anoxic conditions were identified in the laboratory by applying highly selective enrichment conditions with DCM as the only available energy source. Attempts to grow *Dehalobacterium formicoaceticum* and *Dehalobacter* sp. strain

RM1 without DCM have failed, and it is currently unclear what other substrates these organisms can metabolize. Hence, the physiology and function of these bacteria in the environment remain unknown, and their abundance in sediments has not been explored. In addition, the impact of DCM on undisturbed soil microbial communities has not been investigated. The reason why our knowledge about the effects of DCM on microbial communities remains rudimentary to date is the fact that over 99% of soil bacteria have not yet been cultured using traditional culturing techniques (5). The advent of 16S rRNA gene-based molecular biology tools (MBTs) and high-throughput sequencing technologies now allow for the identification and monitoring of non-cultured microorganisms in complex bacterial communities. Environmental changes can elicit rapid alterations to the structure of microbial communities by promoting competition and the preferential growth of community members that are most fit to thrive in the modified conditions. Changes in the relative abundance and phylogenetic composition of a microbial community can also lead to a shift towards metabolic activities in sync with changes in the surrounding environment (4). Next generation sequencing technologies such as the GS FLX pyrosequencing platform by 454 Life Sciences, permit the study of such changes in complex soil microbial communities in response to perturbations.

The objective of this work was to assess the changes in the bacterial community composition of microcosms prepared with Rio Mameyes sediment – a pristine river in Luquillo, Puerto Rico – upon the addition of DCM. Additionally, this study aimed to track the abundance of the DCM-fermenter *Dehalobacter* sp. strain RM1 throughout the enrichment process for DCM degradation by consortium RM.

5.2 Materials and Methods

5.2.1 Sediment collection and microcosm set up. Pristine fresh water sediment was collected from Rio Mameyes in Luquillo, Puerto Rico in October 2009 (latitude 18°21'43.9", longitude -65°46'10"). Microcosms were established as previously described (24). Each microcosm received 20 mg L⁻¹ DCM ($\approx 128 \mu\text{M}$ aqueous phase concentration) and triplicate microcosms were incubated statically at room temperature in the dark. Upon consumption of the initial dose of DCM, additional DCM (20 mg L⁻¹) was amended into the microcosm vessels. In total, 10 DCM additions (each of 20 mg DCM L⁻¹) were provided – each addition made after the previous one had been consumed. Microcosms prepared with heat-killed (i.e., autoclaved) slurry and amended with DCM served as control incubations.

5.2.2 Transfer cultures. The initial microcosms prepared with Rio Mameyes sediment served as inocula to start a set of ten serial transfer cultures. Glass serum bottles (160 mL, nominal capacity) containing 96 mL of medium received 4% inocula (vol/vol) and 20 mg L⁻¹ DCM. In total, five to 10 DCM feedings of 20 mg L⁻¹ were provided to each transfer culture vessel.

5.2.3 Analytical methods. DCM was monitored via manual headspace injections (0.1 mL) with a Hewlett-Packard (HP) 6890 gas chromatograph (GC) equipped with an HP-1 Crosslinked Methyl Siloxane column (30 m x 0.32 mm; film thickness, 0.25 μm nominal) and an electron capture detector (ECD). The GC method used for DCM quantification included the inlet, column, oven and detector parameters previously described by Justicia-Leon et al. (24). The method provided a linear detector response for DCM

concentrations from 1.5 μM to 385 μM . Standards were prepared by adding known amounts of DCM to culture vessels containing medium.

5.2.4 DNA extraction. DNA was obtained from 1g of Rio Mameyes sediment using the Mo Bio Power Soil DNA Extraction Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) as described in the manufacturer's instruction manual. DNA was also obtained from 1 mL of culture suspension from one of the initial microcosms prepared with Rio Mameyes sediment, as well as from the 1st, 2nd, 4th, 5th and 10th transfer cultures using the QIAmp DNA Micro Kit (Qiagen, Germantown, MD) for the isolation of genomic DNA from the pelleted cell material with the following modifications: Four μL of RNase A (100 mg mL^{-1}) were added to 200 μL Buffer AL and the samples were incubated at 70 °C for 10 minutes. The isolation of DNA from the initial microcosm and 1st, 2nd, 4th and 10th transfer cultures was performed upon the consumption of 10 consecutive DCM additions to each culture vessel. For transfer culture 5, DNA was obtained after the consumption of 5 DCM amendments.

5.2.5 454 pyrosequencing of 16S rRNA gene amplicons. Bacterial 16S rRNA gene sequences were obtained from Rio Mameyes sediment DNA, as well as from DNA extracted from the initial microcosm and subsequent transfers. Polymerase chain reaction (PCR) was conducted using an Eppendorf 5341 MasterCycler® epgredient system (Eppendorf, Hauppauge, NY) and barcoded primers designed and tested by the Broad Institute to amplify the V3 through V1 region of the bacterial 16S rRNA genes within each sample. Every 20 μL -reaction contained 2 μL of 10X AccuPrime™ PCR Buffer II, 0.75 U of AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen, Grand Island, NY), 10 μL of template DNA and forward and reverse primers at a final

concentration of 500 nM each. The following thermocycling program was used: initial denaturation for 2 min at 95 °C followed by 30 cycles of denaturation at 95 °C for 20 sec, annealing at 56 °C for 30 sec and extension at 72 °C for 1 min 45 sec. Following amplification, the barcoded-PCR products were subjected to gel electrophoresis in a 2% agarose gel. Gel bands of a size consistent with 16S rRNA genes were excised from the agarose gel, cleaned using the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA) following the manufacturer's instructions and quantified using PicoGreen® (Invitrogen, Grand Island, NY). Subsequently, each sample was diluted to a final concentration of 10^9 16S rRNA gene fragments μL^{-1} . The dilutions of 16S rRNA gene amplicons were combined and sequenced using the GS FLX Titanium pyrosequencing platform by 454 Life Sciences (Roche Diagnostics, Indianapolis, IN) as described in the protocol provided by the manufacturer. The sequences obtained via pyrosequencing were analyzed with the pipeline provided by the QIIME v1.04 open-source software package (9). 16S rRNA gene sequences were first separated based on the sample of origin (i.e., sediment, initial microcosm or subsequent transfers) using the barcode assigned to each DNA sample and then denoised and inspected for chimeras using the corresponding modules included in the QIIME software package. Denoised, non-chimeric sequences were then clustered into operational taxonomic units (OTUs) by selecting a 97% nucleotide sequence identity cut off. The taxonomic affiliation of each OTU was determined through the Greengenes database at the genus level (16). Alpha diversity, or within-sample diversity, was calculated via the Phylogenetic Distance (PD) Whole Tree (i.e., phylogenetic) metric using a script available in the QIIME software package.

5.3 Results

5.3.1 DCM degradation in Rio Mameyes microcosms and serial transfer cultures.

In all live Rio Mameyes microcosms, the initial dose of DCM was consumed within 4 weeks of incubation. Nine additional DCM doses of 20 mg L⁻¹ were consumed at increasing rates (Figure 5.1). Conversely, less than 10% of the initial amount of DCM disappeared in control microcosms prepared with heat-killed Rio Mameyes sediment slurry. Serial transfer cultures (4% inocula, vol/vol) also consumed five to 10 consecutive DCM doses of 20 mg L⁻¹, with each successive DCM dose being consumed at a rate faster than previous doses. A sediment-free enrichment culture, designated culture RM, was obtained after three serial transfers. DCM degradation in the RM culture has been characterized (24).

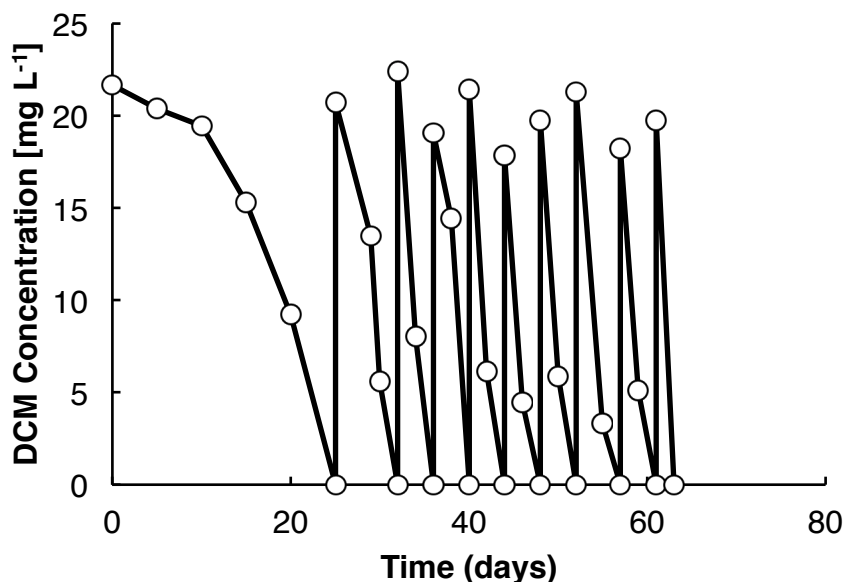


Figure 5.1 DCM degradation in the initial microcosm prepared with Rio Mameyes sediment. Each consecutive dose of DCM (empty circles) was consumed at an increasing rate.

5.3.2 454 pyrosequencing. GS FLX Titanium 454 pyrosequencing produced a total of 49,944 high-quality (i.e., denoised, non-chimeric) barcode-tagged reads of 16S rRNA gene amplicons. The number of sequences originating from different samples varied from 2,371 to 13,781 and the average sequence length was 418 bp. The UCLUST sequence-clustering algorithm was used to align all high-quality sequences and define OTUs abiding by a cut-off of 97% nucleotide sequence identity. Altogether, 2,433 OTUs were defined for the different samples – 1,316 OTUs for the Rio Mameyes sediment sample, 455 OTUs for the initial microcosm, 251 OTUs for the 1st transfer culture, 186 OTUs for the 2nd transfer culture, 292 OTUs for the 4th transfer culture, 187 OTUs for the 5th transfer culture, and 195 OTUs for the 10th transfer culture. Approximately 21.0% of all OTUs (512 OTUs) could not be assigned to a particular phylum by the Greengenes 16S rRNA gene database and were therefore classified as unidentified (i.e., Root). The remaining OTUs grouped into 46 phyla, 13 of which contained 1.0% or more of the OTUs, as follows: *Proteobacteria* with 480 OTUs (19.7%), *Firmicutes* with 289 OTUs (11.9%), *Chloroflexi* with 265 OTUs (10.9%), *Bacteroidetes* with 182 OTUs (7.5%), *Acidobacteria* with 122 OTUs (5.0%), *Planctomycetes* with 110 OTUs (4.5%), *Actinobacteria* with 88 OTUs (3.6%), *Spirochaetes* with 72 OTUs (3.0%), *Nitrospirae* with 38 OTUs (1.6%), *Synergistetes* with 37 OTUs (1.5%), *Verrucomicrobia* with 31 OTUs (1.3%), *Gemmatimonadetes* with 25 OTUs (1.0%), and *Chlorobi* with 25 OTUs (1.0%).

Following sequence clustering and OTU assignment, analysis of alpha diversity (i.e., within sample diversity) based on species richness estimation using the PD Whole Tree metric, and a calculation of simulated sequencing effort were used through the QIIME pipeline to obtain rarefaction curves. All sequenced samples except for the Rio

Mameyes sediment demonstrated rarefaction curves that reached saturation (Figure 5.2), revealing that the sequencing depth was sufficient to describe patterns in bacterial diversity for the enriched samples.

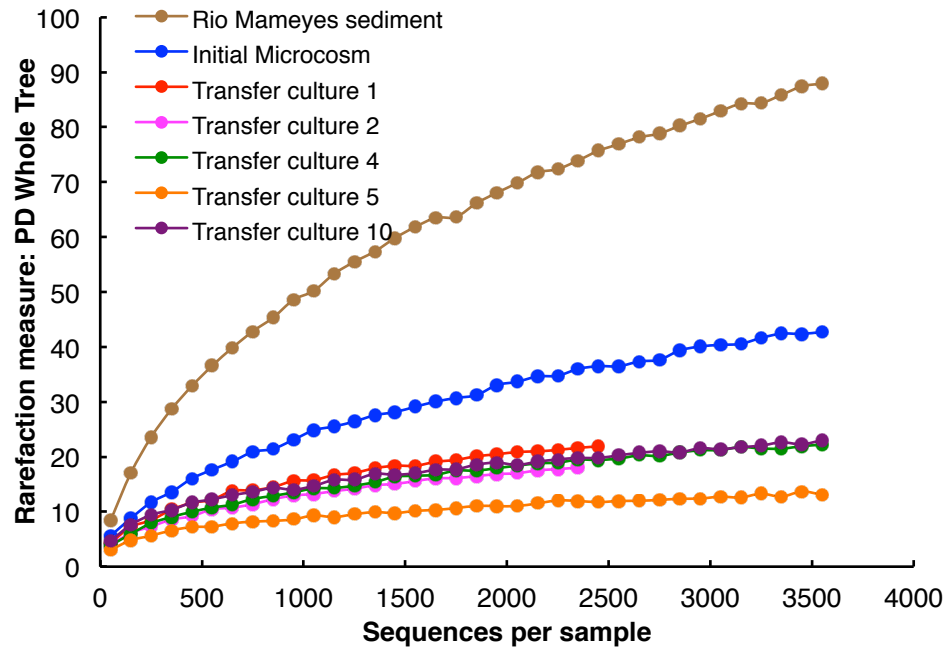


Figure 5.2 Rarefaction curves computed using the phylogenetic diversity (PD) whole tree quantitative metric. The V13 region of the bacterial population in the pyrosequenced samples was sequenced with sufficient depth for all samples except the sediment.

5.3.3 DCM-induced changes of bacterial community structure in Rio Mameyes microcosms and serial transfer cultures

Among the 13 phyla containing 1.0% or more of all OTUs, the *Acidobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Planctomycetes* and *Verrucomicrobia* were represented almost exclusively in the Rio Mameyes sediment sample and the initial microcosm, but mostly absent from transfer cultures (Figure 5.3). OTUs assigned to the

Chlorobi phylum were identified in the Rio Mameyes sediment, the initial microcosm and transfer cultures 1, 2, 4 and 5, but no *Chlorobi* representatives were associated with the 10th transfer culture (Figure 5.3). On the contrary, representatives of the *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Bacteroidetes*, *Spirochaetes*, *Nitrospirae* and *Synergistetes* phyla were present in all of the assayed DNA samples – from the Rio Mameyes sediment to the 10th transfer culture (Figure 5.3).

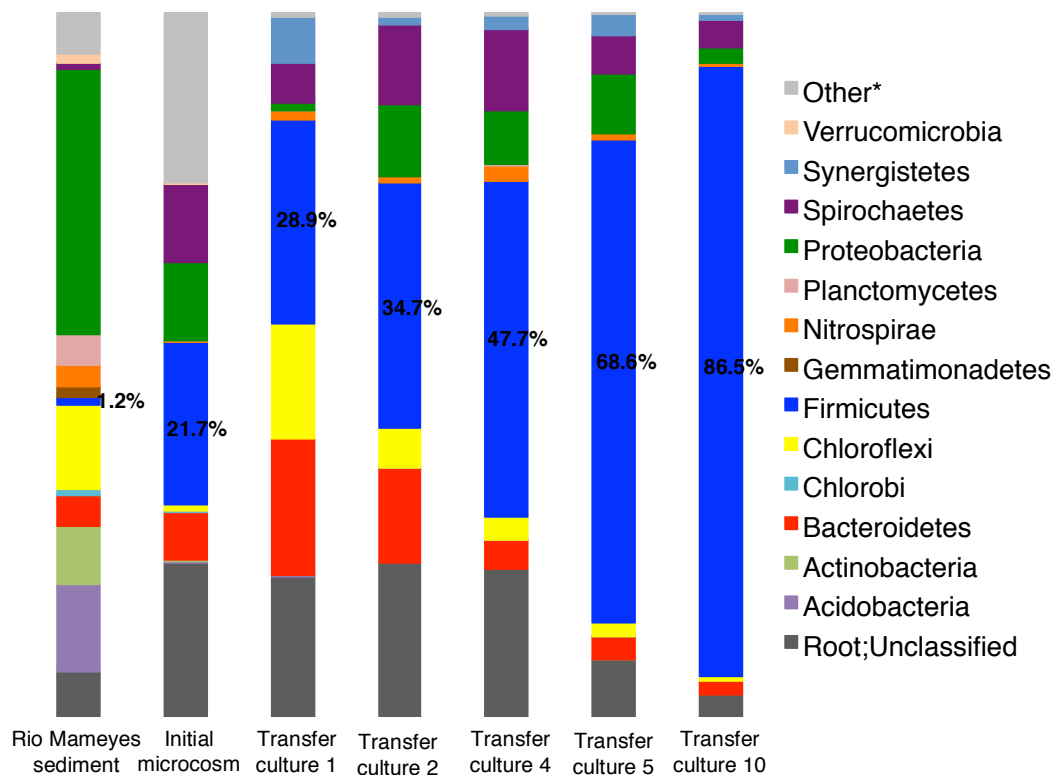


Figure 5.3 Shift in bacterial community composition (at the phylum level) as the enrichment process for DCM degradation progressed. Representatives from the *Firmicutes* phylum show the largest increase in abundance from the Rio Mameyes sediment sample to the 10th transfer culture. Sequences assigned to Root; unclassified could not be assigned to any particular phylum by the QIIME software package using the Greengenes 16S rRNA gene database.

*Other phyla: *ABY1_OD1*, *AC1*, *AD3*, *Armatimonadetes*, *BRC1*, *Caldiserica*, *Cyanobacteria*, *Elusimicrobia*, *Fusobacteria*, *GAL15*, *GN02*, *GN04*, *GOUTA4*, *HDBW-WB69*, *Hyd-2412*, *KSB1*, *Lentisphaerae*, *MVP-15*, *NC10*, *OP11*, *OP3*, *OP8*, *SBR1093*, *SM2F11*, *SPAM*, *TG3*, *TM6*, *TM7*, *Tenericutes*, *Thermi*, *WS1*, *WS3*, *ZB2*.

5.3.3.1 Profile for the Proteobacteria phylum: Association of Alpha-, Beta-, and Gammaproteobacteria with Rio Mameyes sediment and transient enrichment of Delta- and Epsilonproteobacterial representatives upon exposure to DCM

OTUs assigned to the *Proteobacteria* phylum included representatives from the *Alpha*-, *Beta*-, *Delta*-, *Epsilon*- and *Gamma*- classes. The sequences identified as *Alpha*-, *Beta*- and *Gammaproteobacteria* were mainly associated with the Rio Mameyes sediment and, in a few instances, with the initial microcosm (Figure 5.4A). Most bacterial representatives from these *Proteobacteria* classes had a relative abundance of less than 1.0% in the Rio Mameyes sediment. *Rhodoplanes*, *Azohydromonas*, *Nordella* and *Pedomicrobium* genera were exceptions, accounting for 3.7%, 1.4%, 1.2% and 1.1% of the sequences obtained from the Rio Mameyes sediment DNA sample, respectively. Unclassified representatives from the *Burkholderiales* class and the *Bradyrhizobiaceae* and *Rhodospirillaceae* families also accounted for at least 1.0% of all sequences obtained from Rio Mameyes sediment, with 1.0%, 1.5% and 1.7% relative abundances, respectively.

Deltaproteobacterial sequences exhibited the highest relative abundance in the 4th transfer culture, with representatives from the *Geobacter* (2.1%), *Syntrophus* (1.9%) and *Desulfovibrio* (1.7%) genera comprising 5.7% of all the reads obtained for the transfer (Figure 5.4A). The 10th transfer culture demonstrated a decrease in the relative abundance of *Deltaproteobacteria* with only 1.1% of the sequences obtained from this transfer cultures identified as *Geobacter* (0.5%), *Syntrophus* (0.4%) and *Desulfovibrio* (0.2)% (Figure 5.4A). Members from the *Geobacter* genus are known to play an important role as Fe(III)-reducing bacteria in sedimentary environments (12, 29). *Syntrophus* representatives produce organic acids and H₂ from benzoate, crotonate,

butyrate, hexanoate and other salts, and necessitate a close association with a hydrogenotroph for growth (23, 35). Alternatively, *Desulfovibrio* spp. have been described as sulfate-reducing bacteria (46).

Two representatives from the *Epsilonproteobacteria* – *Sulfurospirillum* and *Sulfuricurvum* – demonstrated transient enrichment upon exposure to DCM. Sequences identified as representatives from the *Sulfurospirillum* genus accounted for 7.3% of all the pyrosequencing reads obtained for the initial microcosm, but were absent from all other pyrosequenced samples (Figure 5.4A). Similarly, *Sulfuricurvum* representatives made up 4.8% of all the reads obtained for the 2nd transfer culture, but decreased to 0.3% of the reads from the 10th transfer culture (Figure 5.4A). Bacteria belonging to the *Sulfurospirillum* genus have demonstrated capabilities for fumarate fermentation and dissimilatory nitrate reduction to ammonia, as well as the ability to respire alternative electron acceptors under heterotrophic conditions (8). All known *Sulfuricurvum* spp., on the other hand, have been characterized as sulfur-oxidizing bacteria (26).

5.3.3.2 Profile for the Firmicutes phylum: DCM-induced enrichment of Dehalobacterium, Acetobacterium and Dehalobacter genera

OTUs identified as belonging to the *Clostridiales* order of *Firmicutes* exhibited the highest relative abundances per sample after DCM exposure through the enrichment process. Phylotypes from the *Clostridiales* order made up 0.7%, 21.2%, 28.1%, 33.9%, 44.5%, 69.0% and 78.2% of the sequences obtained from the Rio Mameyes sediment sample, the initial microcosm, and the 1st, 2nd, 4th, 5th and 10th transfer cultures, respectively. In particular, sequences associated with the *Acetobacterium* and *Dehalobacter* genera reflected the increase in *Clostridiales* representatives.

Acetobacterium representatives accounted for 1.6% of the reads obtained from the 1st transfer culture and 17.4% of the reads obtained from the 10th transfer culture (Figure 5.4B). For all other pyrosequenced samples, *Acetobacterium* sequences were either undetected or remained at 0.1% relative abundance. A Basic Local Alignment Search Tool (BLAST) analysis of the pyrosequencing reads identified as *Acetobacterium* against the NCBI-nr database revealed that the *Acetobacterium* representatives in the 10th transfer culture most closely resembled *Acetobacterium wieringae*, a bacterium that grows chemolithotrophically with H₂ and CO₂ producing acetate as sole end product (7). Representatives of the *Dehalobacter* genus were undetected in the Rio Mameyes sediment, but demonstrated a significant increase in relative abundance in all other pyrosequenced samples through enrichment with DCM (Figure 5.4B). Sequences identified as *Dehalobacter* accounted for 0.2%, 1.5%, 15.1%, 26.8%, 65.9% and 59.3% of all the reads obtained from the initial microcosm and the 1st, 2nd, 4th, 5th and 10th transfer culture, correspondingly. All pyrosequencing reads identified as *Dehalobacter* formed one monophyletic group that best matched the nearly full-length 16S rRNA gene sequences of *Dehalobacter* sp. strain RM1 in the NCBI-nr database (Figure 5.5). *Dehalobacter* sp. strain RM1 has been shown to grow concomitant with the fermentation of DCM by consortium RM (24).

Upon the addition of DCM, transient enrichment was observed for OTUs assigned to the *Desulfosporosinus*, *Acidaminobacter* and *Dehalobacterium* genera of the *Clostridiales* (Figure 5.4B). *Desulfosporosinus* representatives comprised 14.6% of the reads associated with the initial microcosm, but were undetected in all other samples. Species belonging to the *Desulfosporosinus* genus have been commonly described as sulfate-reducing bacteria with metal reduction capabilities (41, 43). Alternatively,

representatives from the *Acidaminobacter* genus were undetected in Rio Mameyes sediment and the initial microcosm, but exhibited 1.3%, 3.7%, 0.5%, 0.4% and 0.4% relative sequence abundance in the 1st, 2nd, 4th, 5th and 10th transfer cultures, respectively. Members of the *Acidaminobacter* genus exhibit a chemoorganotrophic fermentative metabolism, produce acetate as major fermentation product and depend on the presence of H₂-utilizing organisms in order to thrive (44). Lastly, the *Dehalobacterium* genus was absent from the Rio Mameyes sediment sample and the 10th transfer culture, but was characterized by pyrosequencing reads that made up 0.3%, 10.6%, 12.1%, 14.5% and 0.3% of the reads obtained from the initial microcosm and the 1st, 2nd, 4th and 5th transfer cultures, respectively. It is important to note that the only *Dehalobacterium* species in the NCBI-nr database (or any other 16S rRNA gene database) to date is *Dehalobacterium formicoaceticum*, a known DCM fermenter described by Mägli et al (31). A BLAST of the pyrosequencing reads identified as *Dehalobacterium* against the NCBI-nr database revealed that these reads shared 89-93% nucleotide identity with the 16S rRNA gene sequence for *Dehalobacterium formicoaceticum*.

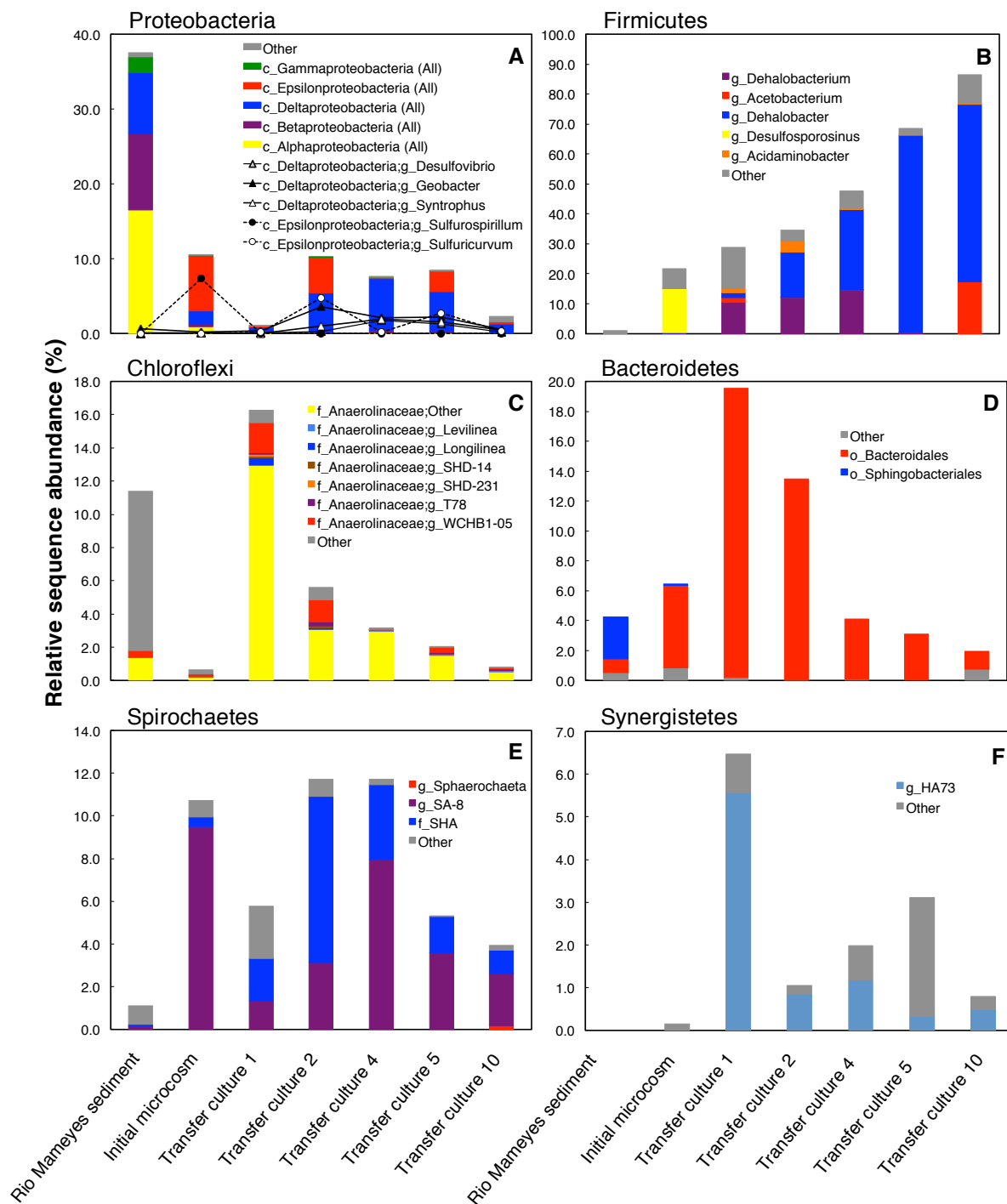


Figure 5.4 Shift in bacterial community composition (within phyla) as the enrichment process for DCM degradation progressed. (A) *Proteobacteria*, (B) *Firmicutes*, (C) *Chloroflexi*, (D) *Bacteroidetes*, (E) *Spirochaetes*, (F) *Synergistetes*.



Figure 5.5 V13-region 16S rRNA reads from transfer culture 10 identified as *Dehalobacter* sp. by the QIIME software package the Greengenes 16S rRNA gene database. A Basic Local Alignment Search (BLAST) was performed with the obtained *Dehalobacter* sp. sequences against the NCB1-nr database and closest phylogenetic hits were used for alignment and phylogeny. Alignment was performed using ClustalW in the Mega v.5.05 software interface with the following parameters: For pairwise alignment - Gap opening penalty: 15, Gap extension penalty: 6.66; For multiple alignment – Gap opening penalty: 15, Gap extension penalty: 6.66; DNA weight matrix: IUB; Transition weight: 0.5. The alignment was trimmed to ~171 nt (with gaps) so that phylogeny could be constructed using one common segment from all of the sequences. A maximum likelihood phylogenetic tree was constructed using TreeExplorer from the Mega v5.05 software interface. The resulting maximum likelihood phylogenetic tree was edited using FigTree V1.3.1 software.

5.3.3.3 Profile for the *Chloroflexi* phylum: Transient enrichment of members from the *Anaerolinaceae* family along the enrichment process for DCM degradation

OTUs representing members of the *Anaerolinaceae* family of the *Chloroflexi* accounted for 9.4%, 0.4%, 16.3%, 5.6%, 2.9%, 1.8% and 0.8% of the sequences obtained from the Rio Mameyes sediment sample, the initial microcosm and the 1st, 2nd, 4th, 5th and 10th serial transfer cultures, correspondingly. The high relative sequence abundance in transfer culture 1 (16.3%) was explained by the presence of sequences belonging to unclassified genera of the *Anaerolinaceae* family (13.7%), as well representatives from the *Longilinea* genus (0.5%) and the WCHB1-05 (1.8%), T78 (0.1%), SHD-231 (0.1%) and SHD-14 (0.1%) groups of *Chloroflexi* (Figure 4C). For the 10th transfer culture in particular, representatives from the *Levilinea* (0.1%), T78 (0.1%) and WCHB1-05 (0.1%) groups, as well as unclassified genera (0.5%), encompassed the diversity assigned to the *Anaerolinaceae* family (Figure 5.4C). To date, the only cultured representatives belonging to the *Longilinea* and *Levilinea* genera are *Longilinea arvoryzae* and *Levilinea saccharolytica*, strictly anaerobic, filamentous bacteria. *Longilinea arvoryzae* was isolated from rice paddy soil and has been shown to grow in the presence of yeast extract with xylose, raffinose, sucrose, xylan, pectin and peptone as substrates (50). *Levilinea saccharolytica*, isolated from sludge granules treating high-strength organic wastewaters, can produce acetate, formate, H₂ and trace amounts of lactate via the fermentation of sugars (e.g. glucose) also in the presence of yeast extract (51). Conversely, members of the T78 clade of the *Chloroflexi* exhibit a wide range of physiological capacities. For example, *Dehalococcoides ethenogenes* strain 195, one isolate belonging to the T78 clade, grows exclusively via the oxidation of H₂ concomitant with the reductive dechlorination of chlorinated hydrocarbons (14, 32); however, other isolates belonging to the same clade, have demonstrated sugar (i.e., glucose and

fructose) fermentation capabilities (40). Members of the T78 clade of the *Chloroflexi* have also been detected in hydrothermal springs, soil, wastewaters and subsurface environments (14, 21, 22). Lastly, the WCHB1 and SHD groups of *Chloroflexi* consist of uncultured representatives that have been identified in sediments contaminated with petroleum, methylmercury and chlorinated solvents (3, 17, 48, 52).

5.3.3.4 Profile for the Bacteroidetes phylum: Transient enrichment of representatives from the Bacteroidales order after DCM exposure

Most of the *Bacteroidetes* representatives identified from the pyrosequencing reads grouped into two orders, *Sphingobacteriales* and *Bacteroidales*. OTUs classified as *Sphingobacteriales* were only associated with the Rio Mameyes sediment and/or the initial microcosm; no *Sphingobacteriales* were identified in any of the transfer cultures (Figure 5.4D). Representative OTUs from the *Bacteroidales* order were identified in all of the samples and accounted for 0.9%, 5.5%, 19.3%, 13.5%, 4.0%, 3.1% and 1.2% of all the pyrosequencing reads obtained from the Rio Mameyes sediment sample, the initial microcosm and transfer cultures 1, 2, 4, 5 and 10, respectively (Figure 5.4D). Unfortunately, the module for assigning taxonomy included in the QIIME software package (using the Greengenes 16S rRNA gene database) could not assign the reads to particular genera within the *Bacteroidales* order. Although the detection of bacteria belonging to the *Bacteroidales* order has historically been associated with fecal contamination, environmental sequences identified in several chlorinated-solvent degrading enrichment cultures have also been linked to *Bacteroidales* representatives as closest phylogenetic relatives in the available 16S rRNA gene databases (6, 47).

5.3.3.5 Profile for the Spirochaetes phylum: Transient enrichment of SA-8 and SHA representatives after DCM exposure in Rio Mameyes derived microcosms and serial transfer cultures

OTUs assigned by the Greengenes 16S rRNA gene database to the *Spirochaetes* phylum demonstrated an increase in relative sequence abundance in the initial microcosm prepared with sediment material from Rio Mameyes and in several subsequent serial transfer cultures. Sequences identified as belonging to the SA-8 group of *Spirochaetaceae* comprised 0.1%, 9.5%, 1.3%, 3.1%, 7.9%, 3.6% and 2.5% of the sequences acquired from the Rio Mameyes sediment sample, the initial microcosm and the 1st, 2nd, 4th, 5th and 10th transfer culture, respectively (Figure 5.4E). Alternatively, representatives from the SHA group of *Spirochaetes* (particularly those denoted W22) made up 1.9%, 7.4%, 3.4%, 1.7% and 1.1% of the sequences collected for transfer cultures 1, 2, 4, 5 and 10, correspondingly, but were absent from Rio Mameyes sediment and the initial microcosm (Figure 5.4E). Both phylogenetic designations, SA-8 and SHA, are used to describe uncultured clones that have been detected in the anoxic sediments of hypersaline ponds in the Mediterranean salterns (SA-8) or in a dichloropropane-dechlorinating enrichment culture (SHA) (34). Interestingly, representatives from the *Sphaerochaeta* genus (0.2% relative sequence abundance) were only identified in the 10th transfer culture. *Sphaerochaeta* isolates have been obtained from reductively dechlorinating consortia and were recently characterized as strictly fermentative and anaerobic organisms that produce ethanol, acetate and formate as major end products from hexose and pentose fermentation (38).

5.3.3.6 Profile for the Nitrospirae phylum: Presence of representatives from the Thermodesulfovibrionaceae family in all pyrosequenced DNA samples

OTUs assigned to the *Thermodesulfovibrionaceae* family of the *Nitrospirae* phylum were obtained from the DNA extracted from the initial microcosm prepared with Rio Mameyes sediment material, as well as from subsequent transfer cultures.

Thermodesulfovibrionaceae-associated reads were not detected in the Rio Mameyes sediment sample, but comprised 1.2%, 0.9% and 0.8% of all reads obtained for the initial microcosm, the 1st transfer culture, the 2nd transfer culture and the 5th transfer culture, respectively. The 4th transfer culture derived from the Rio Mameyes sediment microcosms contained the highest number of OTUs classified as

Thermodesulfovibrionaceae, with such sequences contributing to 2.3% of all the pyrosequencing reads obtained for the transfer culture. Conversely, only 0.4% of the reads obtained from the 10th transfer culture were identified as

Thermodesulfovibrionaceae (or *Nitrospirae*). Bacterium DCE29 was the closest phylogenetic relative of all of the *Thermodesulfovibrionaceae* representatives in transfer culture 10. Sequence type DCE29 was described by Gu et al. as an uncultured clone obtained from a *cis*-dichloroethene (DCE)-degrading enrichment culture and characterized via restriction fragment length polymorphism (RFLP) (20).

5.3.3.7 Profile for the Synergistetes phylum: Transient enrichment of the HA73 phylotype in serial transfer cultures upon exposure to DCM

OTUs assigned to the *Synergistetes* phylum demonstrated an increase in relative sequence abundance in the initial microcosm prepared with Rio Mameyes sediment (Figure 5.4F). Responsible for the increase were pyrosequencing reads associated with the HA73 phylotype of the *Dethiosulfovibrionaceae* family of *Synergistetes*, accounting

for 5.6% of the reads obtained from the DNA extracted from the initial microcosm. Representatives from the HA73 phylotype decreased in relative abundance in subsequent transfer cultures and only 0.5% of the sequences obtained from transfer 10 were still assigned to HA73. Godon et al. described HA73 as a bacterial clone obtained from community analysis performed to an anaerobic digester (19).

5.4 Discussion

DCM degradation under anoxic conditions has been attributed to the bacterial genera *Dehalobacterium* and *Dehalobacter*; however, the prevalence of DCM in anoxic groundwater environments still poses serious environmental concerns (24, 31). The prevalence and recalcitrance of DCM in many contaminated sites across the U.S. suggests that the microorganisms known to degrade DCM anaerobically are either absent from these sites or not performing the physiological function for which they are known. A recent microcosm study demonstrated that bioaugmentation of DCM-contaminated sites with a DCM fermenting consortia resulted in complete detoxification of DCM (Justicia-Leon et al., submitted Environ. Sci. Technol., 2012). Although bioaugmentation with consortium RM proved to be effective in anoxic microcosms amended with DCM, a better understanding of the effects of DCM on the composition and functionality of microbial communities is essential for designing and successfully implementing remediation strategies at contaminated sites.

Based on the barcoded pyrosequencing results obtained in this study, the bacterial community contained in sediment collected from Rio Mameyes exhibited a drastic shift in composition and relative abundance as the enrichment process for DCM degradation

progressed. Among the 13 most prevalent phyla, representatives from the *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Bacteroidetes*, *Spirochaetes* and *Synergistetes* phyla were transiently enriched (Figure 5.4). In particular, species from the *Geobacter*, *Syntrophus* and *Desulfovibrio* genera of *Deltaproteobacteria* and from the *Sulfurospirillum* and *Sulfuricurvum* genera of *Epsilonproteobacteria* demonstrated to be temporarily augmented in the 2nd, 4th and 5th transfer cultures, but decreased in relative abundance by the 10th transfer culture derived from Rio Mameyes sediment. In a similar manner, representative genera from the *Chloroflexi* (i.e., *Anaerolinaceae* family), *Bacteroidetes* (i.e., *Bacteroidales* order), *Spirochaetes* (i.e., SA-8 and SHA phylotypes) and *Synergistetes* (i.e., *Dethiosulfovibrionaceae* family) phyla increased in relative abundance during the earlier stages of the enrichment process for DCM degradation, but had already decreased to below 2.5% relative sequence abundance by the 10th transfer culture derived from Rio Mameyes sediment. Known representatives of the bacterial groups that became transiently enriched upon DCM exposure share capabilities for sulfate, sulfur and metal metabolism or the ability to ferment a variety of organic acids and esters – physiologies frequently associated with bacteria in ‘pristine’ sedimentary environments. The physiologies associated with the bacterial groups that were transiently enriched during the enrichment process for DCM degradation suggest that these bacteria might have thrived in the highly-selective, DCM-impacted environment created in the culture vessels by utilizing substrates (other than DCM) associated with the Rio Mameyes sediment. Carbohydrates, amino acids and metals bound to river sediment, as well as sulfate from the weathering of rocks and minerals or from acid rainfall, could have been used by these microorganisms as substrates for growth.

The *Dehalobacterium* genus also became abundant in serial transfer cultures 1 through 4, reaching a maximum relative abundance of 14.5% in transfer culture 4 (Figure 5.4B). Interestingly, the only known representative of the *Dehalobacterium* genus is *Dehalobacterium formicoaceticum*, a homoacetogenic bacterium reported to ferment DCM to acetate, formate and inorganic chloride (31). The increase in sequences identified as *Dehalobacterium* in the 1st, 2nd and 4th transfer cultures suggests that *Dehalobacterium formicoaceticum* was present in the Rio Mameyes sediment and implies that it might have acted as a key player in the fermentation of DCM in such transfer cultures. It is important to note, however, that the relative abundance of *Dehalobacterium* sequences decreased significantly (to 0.3%) in the 5th transfer culture derived from Rio Mameyes sediment and *Dehalobacterium* sequences were not detected in the 10th transfer culture. The decrease in the abundance of *Dehalobacterium* during the continued enrichment with DCM could have been caused by interspecific competition with other organisms that might have been better suited at degrading DCM in the conditions provided by the transfer-culture environment. One such organism might have been the recently described DCM-fermenting *Dehalobacter* sp. strain RM1 (24). Representatives from the *Dehalobacterium* and *Dehalobacter* genera converged in transfer cultures 1, 2 and 4 derived from Rio Mamayes sediment. *Firmicutes* are known to be important players in the horizontal gene transfer (HGT) of metabolic genes in environmental settings and niche overlapping (such as the convergence of *Dehalobacterium* and *Dehalobacter* representatives) might catalyze exchange of genes within groups in close association (10, 11, 42). Since *Dehalobacter* spp. were solely characterized as dehalorespirers until recently, it would be fascinating to investigate if the capability to ferment DCM was acquired through horizontal gene transfer from the long-known DCM-fermenting *Dehalobacterium formicoaceticum*.

Only representatives from the *Firmicutes* phylum demonstrated a progressive increase in relative abundance from the Rio Mameyes sediment sample to the 10th (and most enriched) transfer culture. In particular, species from the *Acetobacterium* and *Dehalobacter* genera shifted from undetected in the Rio Mameyes sediment sample to a relative abundance of 17.4% and 59.3%, respectively, in the 10th transfer culture, denoted as consortium RM. These results strongly suggest an involvement of members from the *Acetobacterium* and *Dehalobacter* genera – specifically *Acetobacterium wieringae* and *Dehalobacter* sp. strain RM1 – in DCM degradation. The marked increase in abundance of sequences identified as belonging to the *Dehalobacter* genus confirms the increase in 16S rRNA gene copies mL⁻¹ of *Dehalobacter* sp. strain RM1 concomitant with DCM fermentation, previously quantified via qPCR by Justicia-Leon et al. (24). The prevalence of *Acetobacterium* representatives in the 10th transfer could indicate a role for interspecies H₂ transfer as part of DCM degradation. Lee et al. quantified low concentrations of formate and compiled circumstantial evidence for H₂ production during DCM fermentation by a *Dehalobacter* sp. in microcosms prepared with subsurface sediments (27). The authors also observed inhibition of DCM fermentation in the presence of H₂ (27). *Acetobacterium wierengae* may possibly be relieving DCM fermentation in consortium RM from H₂ inhibition by rapidly up-taking any H₂ produced and generating acetate (7).

The effect of environmental perturbation in bacterial communities has been widely studied in the past decades; however, few studies have employed MBTs and high throughput sequencing technologies to gain a deeper insight into the changes that occur in a bacterial community through a process of highly-selective enrichment. Ramirez-Saad et al. demonstrated bacterial community shifts in microcosms prepared with peat-

forest soil and amended with 3-chlorobenzoate (3CBA) or 2,5-dichlorobenzoate (2,5DCB) as degradation of these compounds proceeded (37). The authors used 16S rRNA gene PCR amplification and subsequent denaturing gradient gel electrophoresis (DGGE) at different time points throughout the 15-day incubation to show a rapid decrease of microbial diversity and a concomitant shift towards a *Burkholderia*-dominated community (37). More recently, Xu et al. utilized 454 GS FLX barcoded pyrosequencing to study the response of polybrominated diphenyl ethers (PBDE)-degrading microbial communities to changes in the type of electron donor supplied (49). Although the authors did not include serial transfer cultures as part of this study, pyrosequencing analysis revealed differences in the composition of the PBDE-degrading microbial communities supplied with different types of electron donors as compared to an unamended control (49). Interestingly, representatives from the *Bacteroidetes* and *Spirochaetes* phyla increased in abundance in microcosms that received electron donor amendments (49). Calculation of the Shannon-Weaver diversity index (H') for the control PBDE-degrading microcosm, as well as for the five microcosms amended with different electron donors (i.e., methanol, ethanol, acetate, lactate and pyruvate), revealed that the shift in abundance of certain populations was not necessarily correlated with a decrease in microbial diversity through PBDE-degradation (49). Conversely, our results demonstrate a significant decrease in the diversity of the microbial community as the enrichment process for DCM degradation progresses (Figure 5.6). The Shannon-Weaver diversity index associated with the microbial community from the Rio Mameyes sediment dropped from 9.54 for the actual sediment sample to 4.84 for the 10th transfer culture, showing a marked decrease in diversity in response to the highly-selective, DCM-impacted environment generated in the culture vessels.

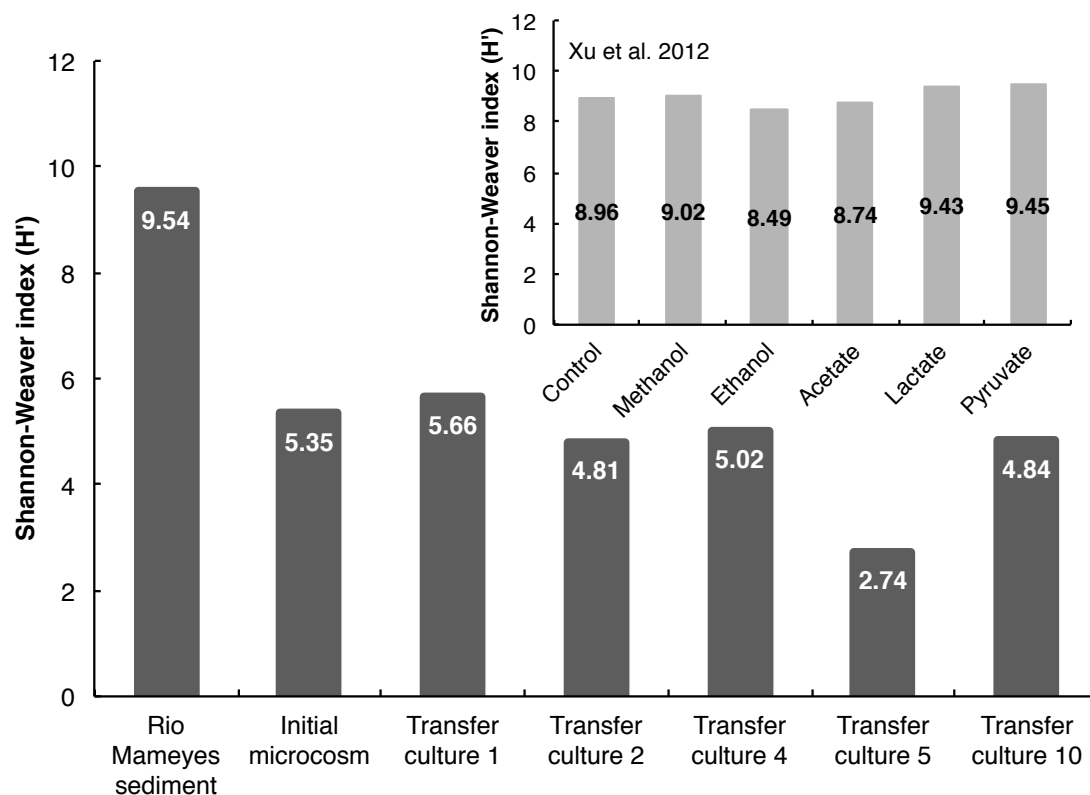


Figure 5.6 Shannon-Weaver diversity index calculation for all pyrosequenced samples in this study compared to the pyrosequenced samples from Xu et al. (49) (in the inset).

The results reported herein provide valuable insights into the effects of DCM in soil microbial communities. Barcoded pyrosequencing allowed for the detailed study of the shifts in microbial community composition during the enrichment process for DCM degradation in consortium RM. Most notably, representatives from the *Firmicutes* phylum (i.e., *Dehalobacter*, *Acetobacterium*, *Dehalobacterium*) demonstrated a significant increase in abundance throughout the enrichment process, revealing the importance of genera from this phylum for the degradation of DCM and suggesting the involvement of such genera in the bioremediation of DCM at contaminated sites. Further analysis of the shift in the composition of functional (i.e., metabolic) genes as DCM

degradation progresses in consortium RM would increased our understanding of the effects of DCM on this particular community. The information obtained from both 16S rRNA (i.e., phylogenetic) and functional (i.e., metabolic) genes examination could prove to be useful for the design and implementation of bioremediation strategies at chlorinated-methane impacted sites.

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CHAPTER 6

DICHLOROMETHANE DEGRADATION IN ANOXIC SEDIMENT MICROCOSMS AND DETECTION OF *DEHALOBACTERIUM FORMICOACETICUM* AND *DEHALOBACTER* SP. STRAIN RM1

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6.1 Introduction

Chlorinated methanes are among the most prevalent contaminants in soils and aquifers. The higher-chlorinated methanes, carbon tetrachloride (CT) and chloroform (CF), can move readily through soil and adsorb only slightly to sediments (1, 2). As a result, any spills or releases of CT and/or CF onto soil rapidly leach into groundwater environments threatening ecosystem and human health. A number of abiotic processes have been associated with the transformation of CT and CF into lesser-chlorinated methanes and other non-chlorinated products. Sulfide and sheet silicates, pyrite, iron(II)-coated goethite, chloride green rust and ferrous sulfide, among other reactive chemical reductants have been shown to catalyze the transformation of CT and CF under anoxic conditions (3, 6, 7, 9, 11, 14, 22, 23, 29, 35, 36). In addition, a recently described *Dehalobacter* sp. contained in consortium Dhb-CF can utilize CF as terminal electron acceptor in a process known as dehalorespiration (or reductive dechlorination) (13). Both the abiotic transformation of CT and CF and the reductive dechlorination of CF by the *Dehalobacter* sp. in consortium Dhb-CF bring about the production of DCM. DCM can be readily degraded by methylotrophic bacteria containing glutathione-dependent

dehalogenases under oxic conditions (5, 16, 20, 21, 25, 37, 44); however, the DCM produced via the abiotic and biotic transformations of CT and CF is released into anoxic, subsurface environments where it has proven to be persistent and recalcitrant.

Dehalobacterium formicoaceticum and *Dehalobacter* sp. strain RM1 are the only anaerobic dichloromethane (DCM)-degrading organisms described to date (19, 32).

Dehalobacterium formicoaceticum grows fermentatively by deriving energy from the oxidation of DCM to acetate in the absence of any exogenous electron acceptor (32-34).

Similarly, *Dehalobacter* sp. strain RM1 ferments DCM to acetate and inorganic chloride in consortium RM – an enrichment culture derived from pristine river sediment (19).

Bioaugmentation with consortium RM of anoxic microcosms prepared with sediment material from a DCM-impacted site achieved complete DCM degradation, revealing the importance of DCM-fermenting organisms for bioremediation strategies (Justicia-Leon et al., submitted to Environ. Sci. Technol., 2012).

For site managers and remediation consultants the quantification of biodegradation potential at a particular site is essential for the design and successful implementation of any remediation strategy. Obtaining information pertaining to the presence and abundance of known DCM-degrading microorganisms is vital before selecting a remediation approach for a DCM-contaminated site. Traditionally, the biodegradation potential of an impacted site has been assessed via microcosm treatability studies or other culture-dependent methods. These laboratory-scale techniques can prove to be very labor-intensive and time-consuming, and will not always yield conclusive results. Culture-independent approaches, such as 16S rRNA gene-based detection and

quantification, have become a reliable alternative for the evaluation of a site's intrinsic capacity for contaminant bioremediation.

16S rRNA gene-based real-time quantitative polymerase chain reaction (qPCR) approaches have been developed for several bacterial genera implicated in the degradation of chlorinated contaminants. For instance, a number of qPCR primers have been designed to target and quantify the 16S rRNA gene copies of *Dehalococcoides* spp. – major players in the complete detoxification of chlorinated ethenes (10, 18, 41). *Dehalococcoides*-specific qPCR approaches have proven to be useful to explore the biodegradation potential at chlorinated ethene-contaminated sites and to establish connections between the presence and abundance of *Dehalococcoides* spp. and *in situ* detoxification (26, 27, 40, 42). Similar approaches are desirable for the detection and quantification of bacteria involved in chlorinated methane degradation and, more specifically, in DCM fermentation.

Herein, we present the design of two qPCR assays to differentially detect and quantify *Dehalobacterium formicoaceticum* and *Dehalobacter* sp. strain RM1. In addition, we report the results of the distribution and abundance of these DCM fermenters across multiple microcosms prepared with sediment material from both pristine and contaminated sites that demonstrated DCM degradation.

6.2 Materials and Methods

6.2.1 Sediment collection and microcosm set up. Pristine fresh water sediment was collected from San Gregorio Creek, CA (latitude 37°19'7.3", longitude -122°17'45.5") and

La Honda Creek, CA (latitude 37°17'50.1", longitude -122°15'39.1") in August 2009, from Green River, UT (latitude 38°59'41", longitude -110°8'36.2"), Colorado River, UT (latitude 38°36'16.9", longitude -109°34'24.7"), San Miguel River, CO (latitude 38°7'32.4", longitude -108°12'24.2"), Uncompahgre River, CO (latitude 38°9'5.6", longitude -107°45'6.4"), Blue Mesa Reservoir, CO (latitude 38°28'14.8", longitude -107°8'42.4"), Moniteau River, MO (latitude 38°58'55.4", longitude -92°33'58.9"), Missouri River, MO (latitude 38°58'21", longitude -92°133'32.2") and Colorado River, CO (latitude 38°40'5.7", longitude -91°33'17.7") in September 2009 and from Rio Mameyes, Luquillo, PR (latitude 18°21'43.9", longitude -65°46'10") and El Seco Beach, Mayagüez, PR in October 2009. Sediment samples were also obtained from two chlorinated methane-contaminated industrial sites – one in northern CA and one in eastern Brazil (both in December 2010) – and from Third Creek, Knoxville, TN – a location known to be impacted with petroleum-based hydrocarbons and chlorinated solvents (March 2011). Two sets of microcosms were established for each sediment sample as previously described with the following modifications: Two pipettable slurries were prepared, one by mixing the sediment samples with anoxic, bicarbonate-buffered (30 mM, pH 7.2) and HEPES-buffered (10 mM, pH 7.2) mineral salts medium and another by mixing the sediment samples with phosphate-buffered (10 mM, pH 7.2) and lactate-amended (5 mM) mineral salts medium (19, 30). Each microcosm received 20 mg L⁻¹ DCM (≈128 μM aqueous phase concentration). Microcosms were incubated statically at room temperature in the dark. Upon consumption of the initial dose of DCM, additional DCM (20 mg L⁻¹) was amended into the microcosm vessels. In total, 5 DCM additions (each of 20 mg DCM L⁻¹) were provided – each addition made after the previous one had been consumed. Microcosms prepared with heat-killed (i.e., autoclaved) slurries and amended with DCM served as control incubations.

6.2.2 Analytical methods. DCM was monitored via manual headspace injections (0.1 mL) with a Hewlett-Packard (HP) 6890 gas chromatograph (GC) equipped with an HP-1 Crosslinked Methyl Siloxane column (30 m x 0.32 mm; film thickness, 0.25 μ m nominal) and an electron capture detector (ECD). The GC method used for DCM quantification included the inlet, column, oven and detector parameters previously described by Justicia-Leon et al. (19). The method provided a linear detector response for DCM concentrations from 1.5 μ M to 385 μ M. Standards were prepared by adding known amounts of DCM to culture vessels containing medium.

6.2.3 DNA extraction and 16S rRNA gene quantification. After the depletion of five consecutive DCM amendments, DNA was obtained from 1 mL of microcosm suspension using the QIAmp DNA Micro Kit (Qiagen, Germantown, MD) for the isolation of genomic DNA from the pelleted cell material with the following modifications: Four μ L of RNase A (100 mg mL⁻¹) were added to 200 μ L Buffer AL and the samples were incubated at 70 °C for 10 minutes. The abundances of 16S rRNA gene copies from the DCM-degrading *Dehalobacterium formicoaceticum* and *Dehalobacter* sp. strain RM1 were measured for each microcosm using qPCR. Primer Express v3.0 software was used to design primer/probe combinations that differentially amplified a 16S rRNA gene fragment of *Dehalobacterium formicoaceticum* or *Dehalobacter* sp. strain RM1. For primer and probe design purposes, *Dehalobacter* 16S rRNA gene sequences, as well as the only 16S rRNA gene sequence available for the *Dehalobacterium* genus, were retrieved from the NCBI-nr database and aligned using ClustalW in MEGA v5.0 software package. The specificity of the primers and probe were evaluated using BLAST analysis and verified experimentally with genomic DNA from the CF-to-DCM-respiring consortium Dhb-CF, *Dehalobacter restrictus* (DSM 9455) and from the DCM-degrading consortium RM

containing *Dehalobacter* sp. strain RM1. The primer and probe combinations were also assayed against a synthetic 1319 bp construct of the *Dehalobacterium formicoaceticum* 16S rRNA gene (Invitrogen Life Technologies, Grand Island, NY). The primers and probes targeting the 16S rRNA gene of *Dehalobacterium formicoaceticum* and *Dehalobacter* sp. strain RM1 are shown in Table 1. qPCR was conducted using an ABI 7500 Fast Real Time PCR System equipped with SDS v2.0.3 software (Applied Biosystems, Carlsbad, CA). Every 20 μ L-reaction contained 10 μ L of 2x Taqman® Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA), 2 μ L of DNA template, and forward and reverse primers and probe at final concentrations of 300 nM each. The following thermocycling program was used: initial denaturation for 2 min at 50 °C and 10 min at 95 °C and 45 cycles of denaturation at 95 °C for 15 sec and annealing and extension at 60 °C for 1 min. Calibration used standard curves based on triplicate qPCR series obtained with 10-fold dilutions of the 16S rRNA genes of the DCM-fermenting *Dehalobacterium* and *Dehalobacter* strains. For this purpose, the 16S rRNA gene of *Dehalobacter* sp. strain RM1 was cloned in *E. coli* as described and added as template DNA to yield reactions spanning a concentration range from 3.38×10^7 copies down to approximately 3 copies (43). The synthetic 16S rRNA gene fragment purchased from Life Technologies was cloned into a pMX vector containing an ampicillin-resistance gene and added as template DNA to yield reactions spanning a concentration range from 3.38×10^7 copies down to approximately 3 copies.

6.3 Results

6.3.1 DCM degradation in live microcosms. In live microcosms established with bicarbonate- and HEPES-buffered mineral salts medium and sediment material from San Gregorio Creek, Green River, El Seco Beach, Rio Mameyes, an industrial site in eastern Brazil and Third Creek, the initial dose of DCM was consumed within 4 weeks of incubation. Similarly, microcosms established with phosphate-buffered, lactate-amended mineral salts medium using sediment material from San Gregorio Creek, La Honda Creek, Colorado River, San Miguel River, Uncompahgre River, Blue Mesa Reservoir, Moniteau River, Missouri River, Casconade River, El Seco Beach, Rio Mameyes, an industrial site in northern CA and Third Creek, the initial dose of DCM was also consumed within 4 weeks of incubation. Five additional DCM doses of 20 mg L⁻¹ were consumed at increasing rates. Conversely, less than 10% of the initial amount of DCM disappeared in control microcosms prepared with heat-killed sediment slurries.

6.3.2 Detection, differentiation and quantification of DCM-fermenting

***Dehalobacterium* and *Dehalobacter* strains.** Primer/probe combinations were designed to quantify *Dehalobacterium formicoaceticum* and *Dehalobacter* sp. strain RM1 using qPCR (Table 6.1). The *Dehalobacter* sp. strain RM1 16S rRNA gene-targeted primer/probe set yielded the expected amplicons, and no amplification was observed when the cloned 16S rRNA genes of *Dehalobacterium formicoaceticum*, the *Dehalobacter* strain in consortium Dhb-CF or *Dehalobacter restrictus* were used as DNA templates. Standard curves for the *Dehalobacter* sp. strain RM1-specific primer and probe combination had a slope of -3.4, a y-intercept of 38, an R² value of 0.99, and an amplification efficiency of 96.9% (Figure 6.2A). Similarly, the primer/probe set designed

for the quantification of *Dehalobacterium formicoaceticum* did not generate fluorescence signals with template DNA of *Dehalobacter* sp. strain RM1 nor the closely related *Dehalobacter* sp. in consortium Dhb-CF or *Dehalobacter restrictus*. Standard curves for quantifying the 16S rRNA gene copies of *Dehalobacterium formicoaceticum* had a slope of -3.2, a y-intercept of 38.6, an R^2 value of 0.99, and an amplification efficiency of 103.6% (Figure 6.2B).

Table 6.1 Primer/probe sets used for 16S rRNA gene-targeted qPCR and qPCR standard curve information.

Target	Primer/Probe Sequence
<i>Dehalobacterium formicoaceticum</i>	Forward Primer 5'-CACCACGAAAGTTGGCAACA Reverse Primer 5'- TTCGGCGACTGCTTCCTT Probe (MGB) 5'-6-FAM- AAGTCGATGAGCGAACC-BHQ1
<i>Dehalobacter</i> sp. strain RM1	Forward Primer 5'-TCCCGCAACGAGCGCAACCCCTATA Reverse Primer 5'-TTTGTACCGGCAGTCTATCCA Probe 5'-6-FAM-CAGTTACCAGCACGTAAAGGTGG-BHQ1

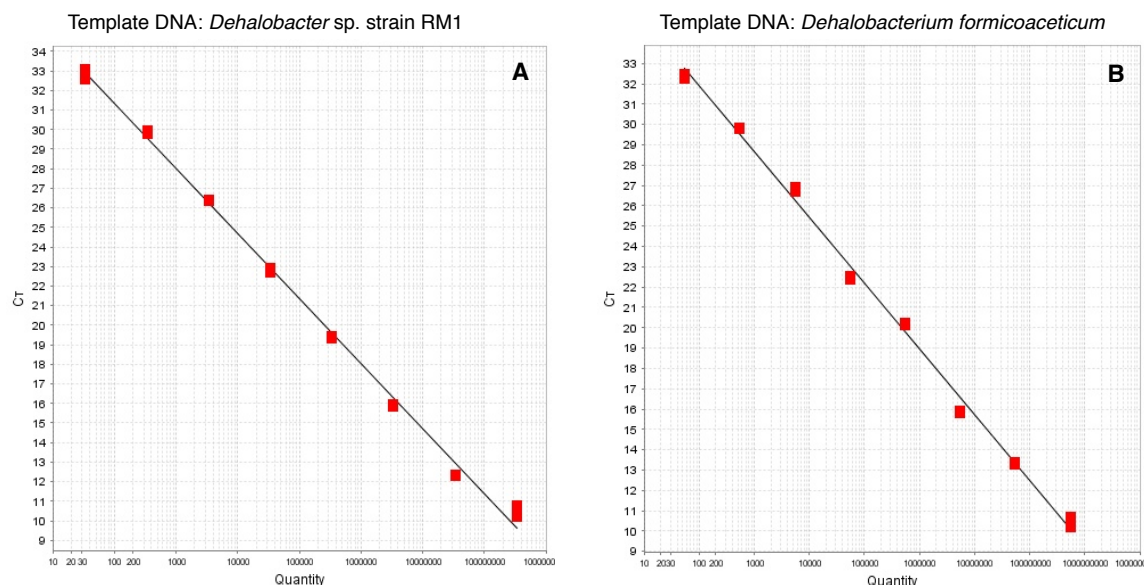


Figure 6.1 Standard curves for primer/probe sets used for 16S rRNA gene-targeted qPCR. (A) Standard curves for the *Dehalobacter* sp. strain RM1-specific primer and probe combination had a slope of -3.4, a y-intercept of 38, an R^2 value of 0.99, and an amplification efficiency of 96.9%. (B) Standard curves for quantifying the 16S rRNA gene copies of *Dehalobacterium formicoaceticum* had a slope of -3.2, a y-intercept of 38.6, an R^2 value of 0.99, and an amplification efficiency of 103.6%

6.3.3 qPCR screening of microcosms that exhibited DCM degradation for

***Dehalobacterium formicoaceticum* and *Dehalobacter* sp. strain RM1.** DNA isolated from microcosms that demonstrated degradation of five consecutive doses of DCM was used as template for qPCR analyses. Each DNA sample was screened for the presence and abundance of the DCM-fermenting *Dehalobacterium* and *Dehalobacter* strains. The recently described *Dehalobacter* sp. strain RM1 was detected in four out of the six microcosms established with bicarbonate- and HEPES-buffered mineral salts medium that exhibited DCM degradation (Table 6.2). DNA from microcosms prepared with sediment material from El Seco Beach, Rio Mameyes and an industrial site in eastern Brazil yielded 1.52×10^5 , 5.37×10^6 and 1.14×10^5 *Dehalobacter* sp. strain RM1 16S rRNA gene copies mL^{-1} , respectively. In addition, the DCM-fermenting *Dehalobacter*

strain was detected in microcosms derived from San Gregorio Creek sediment, but quantification was unattainable because the qPCR amplification signal fell below the concentration range covered by the standard curve for the *Dehalobacter* sp. strain RM1-specific primer/probe set. Interestingly, *Dehalobacter* sp. strain RM1 was only detected in one out of the 13 microcosms established with phosphate-buffered, lactate-amended mineral salts medium that degraded five consecutive doses of DCM. The microcosm derived from sediment collected from an industrial site in northern CA exhibited an abundance of 3.27×10^6 *Dehalobacter* sp. strain RM1 16S rRNA gene copies mL⁻¹. Conversely, *Dehalobacterium formicoaceticum* was detected in two of six DCM-degrading microcosms prepared with bicarbonate- and HEPES-buffered mineral salts medium and in one of the microcosms established with phosphate-buffered, lactate-amended mineral salts medium (Table 6.2). DNA extracted from the microcosm derived from Green River sediment yielded a *Dehalobacterium formicoaceticum* abundance of 7.06×10^4 16S rRNA gene copies mL⁻¹. Both of the microcosms established with Rio Mameyes sediment (in bicarbonate- and HEPES-buffered or in phosphate-buffered, lactate-amended mineral salts medium) contained *Dehalobacterium formicoaceticum* at 2.97×10^5 and 3.07×10^4 , respectively.

Table 6.2 Distribution and abundance of the DCM-fermenting *Dehalobacterium* and *Dehalobacter* strains in microcosms that exhibited DCM degradation. The rows shaded in gray pertain to the results obtained for microcosms established with bicarbonate- and HEPES-buffered mineral salts medium; all other rows contain data obtained from qPCR assays performed on microcosms established with phosphate-buffered, lactate-amended mineral salts medium. ‘ND’ denotes that fluorescence signals were ‘not detected’ in the qPCR assay. ‘Detect/Not quant’ (‘detectable/not quantifiable’) denotes that fluorescence signals were detected for the particular qPCR assay, but where below the concentration range covered by the corresponding standard curve.

Microcosm sediment source	16S rRNA gene copies mL ⁻¹	
	<i>Dehalobacterium formicoaceticum</i>	<i>Dehalobacter</i> sp. strain RM1
San Gregorio Creek, CA	ND	Detect/Not quant
Green River, UT	7.06 x 10 ⁴	ND
El Seco Beach, Mayagüez, PR	ND	1.52 x 10 ⁵
Rio Mameyes, Luquillo, PR	2.97 x 10 ⁵	5.37 x 10 ⁶
Industrial site in eastern Brazil	ND	1.14 x 10 ⁵
Third Creek, TN	ND	ND
San Gregorio Creek, Ca	ND	ND
La Honda Creek, CA	ND	ND
Colorado River, UT	ND	ND
San Miguel River, CO	ND	ND
Uncompahgre River, CO	ND	ND
Blue Mesa Reservoir, CO	ND	ND
Moniteau River, MO	ND	ND
Missouri River, MO	ND	ND
Casconade River, MO	ND	ND
El Seco Beach, Mayagüez, PR	ND	ND
Rio Mameyes, Luquillo, PR	3.07 x 10 ⁴	ND
Industrial site in northern CA	ND	3.27 x 10 ⁶
Third Creek, TN	ND	ND

6.4 Discussion

Although early applications of qPCR were mainly for the detection and quantification of pathogens (4, 8, 17, 28, 31, 38, 39), many recent applications have responded to the analysis of environmental samples. Universal *Eubacteria*- and *Archaea*-specific qPCR primers and probes have been designed to allow for the detection and quantification of representatives from within an entire domain without the need of multiple approaches (12, 15, 24, 41, 45, 46). In addition, particular primer and probe combinations are frequently used to analyze environmental samples for the presence and abundance of specific organisms that perform key roles in geochemical cycles (e.g., methanotrophic bacteria, nitrifying and denitrifying bacteria, sulfate-reducing bacteria, cyanobacteria and xenobiotic-compound degraders, among other classifications) (47). qPCR approaches can be designed to target functional (i.e., metabolic) genes associated with a process of interest or phylogenetic (i.e., 16S rRNA) genes specifically associated with an organism relevant to the process in question. The primer and probe combinations designed in this study selectively target fragments of the 16S rRNA gene of *Dehalobacterium formicoaceticum* and *Dehalobacter* sp. strain RM1, the only anaerobic DCM degraders known to date. *Dehalobacterium formicoaceticum*- and *Dehalobacter* sp. strain RM1-specific primer and probe combinations now allow for the screening of environmental samples for the presence and abundance of these DCM degraders. The detection of *Dehalobacterium formicoaceticum* or *Dehalobacter* sp. strain RM1 at a chlorinated-methane contaminated site may suggest an intrinsic potential for the natural attenuation of DCM. Furthermore, the detection (or lack thereof) of the DCM-fermenting *Dehalobacterium* and *Dehalobacter* strains at a site with recorded persistence of DCM,

may steer site managers and environmental consultants towards biostimulation or bioaugmentation approaches.

In this study, the distribution and commonality of *Dehalobacterium formicoaceticum* and *Dehalobacter* sp. strain RM1 were assessed by means of a novel qPCR approach in microcosms that demonstrated DCM degradation. *Dehalobacterium formicoaceticum* was present in two (out of six) microcosms prepared with bicarbonate- and HEPES-buffered mineral salts medium and in one (out of 13) microcosm established with phosphate-buffered, lactate-amended medium. In a similar way, *Dehalobacter* sp. strain RM1 was detected and successfully quantified in half (three out of six) of the microcosms prepared with bicarbonate- and HEPES-buffered mineral salts medium and in one (out of 13) microcosm established with phosphate-buffered, lactate-amended medium. Since both *Dehalobacterium formicoaceticum* and *Dehalobacter* sp. strain RM1 require bicarbonate for growth (19, 32), their presence (and abundance) in two of the microcosms established with phosphate-buffered mineral salts medium and lacking bicarbonate was unexpected. It is possible that bicarbonate associated with the solids in these two microcosm vessels, as well as CO₂ derived from the fermentation of the lactate amendment, fulfilled the bicarbonate requirement of the DCM-fermenting *Dehalobacterium* and *Dehalobacter* strains. In total, DCM degradation was observed in microcosms derived from 15 distinct sediment samples. Interestingly, *Dehalobacterium formicoaceticum* and/or *Dehalobacter* sp. RM1 were only detected in microcosms established with 5 of these sediment samples. The DCM degradation activity in the remaining microcosms had a definite biological origin, as demonstrated by the persistence of DCM in control microcosms established with autoclaved slurries. These results suggest that our knowledge regarding the diversity of DCM-degrading organisms

remains rudimentary and that DCM degradation in sediments can be carried out by yet unidentified microorganisms.

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CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

Bioaugmentation with microbial consortia containing organohalide-respiring bacteria has emerged as a valuable bioremediation approach at sites impacted with chlorinated solvents. The use of bioaugmentation inocula containing organohalide-respiring *Dehalococcoides mccartyi* strains is now a widely accepted strategy for the remediation of chlorinated ethenes. A similar approach is desirable for sites impacted with chlorinated methanes (i.e., CT, CF, DCM and CM), since these compounds have also become widespread and recalcitrant contaminants that pose risks to human and ecosystem health. The successful implementation of biological remedies at chlorinated methane-impacted sites requires the elucidation of the microbiology contributing to the degradation of chlorinated methanes, as well as the environmental factors controlling such microbial activities. In addition, tools that can efficiently monitor the chlorinated methane-degradation process *in situ* have to be designed and evaluated.

This research effort derived a sediment-free enrichment culture, designated as consortium RM, from pristine Rio Mameyes sediment. Detailed investigation of consortium RM revealed genera from the *Firmicutes* phylum as key players in the degradation of DCM to non-toxic products. Results obtained from high-throughput sequencing of samples collected along the enrichment process with DCM as the sole substrate indicated a significant increase in abundance for representatives of the *Dehalobacterium*, *Acetobacterium* and *Dehalobacter* genera. Additional exploration by

means of 16S rRNA gene-targeted analyses identified *Dehalobacter* sp. strain RM1 as a DCM-fermenting organism in consortium RM.

The application of molecular biological tools (MBTs) and other *in situ* monitoring techniques has greatly improved the ability of scientists and environmental engineers to establish cause-and-effect relationships between microbial activity and contaminant detoxification. In the present work, novel MBTs (i.e., qPCR protocols) were developed, optimized and employed to specifically detect and quantify the DCM-fermenting organisms *Dehalobacterium formicoaceticum* and *Dehalobacter* sp. strain RM1. These new MBTs were essential for determining the presence and abundance of the known DCM-degrading organisms in microcosms derived from sediment and aquifer materials that demonstrated DCM-degrading activity, and for exploring the distribution of DCM degraders in the environment. The *Dehalobacter* sp. strain RM1-specific qPCR approach was also used to monitor the abundance of this organism as DCM degradation proceeded.

In addition, compound specific isotope analysis (CSIA) was utilized to obtain ^{13}C enrichment factors associated with DCM degradation by consortium RM. The significant fractionation associated with DCM degradation by consortium RM indicated that CSIA can monitor DCM transformation/degradation. The tools developed herein may prove useful for evaluating microbial remedies (e.g., biobarriers, monitored natural attenuation, biostimulation and/or bioaugmentation) at chlorinated methane-impacted sites.

The research detailed in this dissertation aimed to explore microbes and monitoring tools for anaerobic chlorinated methane bioremediation. The results of this study suggest several areas of future research:

- At present, the reaction mechanisms employed by *Dehalobacter* sp. strain RM1 to degrade DCM remain unknown. The calculation of consortium RM's specific ^{13}C enrichment factors and the estimation of the kinetic isotope effect (KIE) as DCM degradation proceeds, suggest that the reaction follows an $\text{S}_{\text{N}}1$ -type mechanism; however, additional exploration is warranted to elucidate the pathway(s) by which DCM is fermented by *Dehalobacter* sp. strain RM1.
- As reported herein, the design and optimization of 16S rRNA-gene targeted qPCR protocols aided in the differential detection and quantification of the two known DCM fermenters - *Dehalobacter* sp. strain RM1 and *Dehalobacterium formicoaceticum*. The identification of functional (i.e., metabolic) genes involved in DCM degradation would enhance phylogenetic (i.e., 16S rRNA gene-based) approaches for monitoring of DCM degradation *in situ*.
- Currently, only two organisms are known to degrade DCM anaerobically. A screening for *Dehalobacter* sp. strain RM1 and *Dehalobacterium formicoaceticum* in 15 microcosms established with distinct sediment samples that demonstrated DCM degradation activity revealed that these known DCM degraders were only present in about one-fourth of the microcosms. These results suggest that yet unidentified microorganisms contribute to DCM degradation in sediments. Further investigation of the microbiology associated with DCM degradation in the microcosms that did not contain *Dehalobacter* sp. strain RM1 and/or *Dehalobacterium formicoaceticum* 16S rRNA gene sequences could possibly uncover novel DCM-degrading microorganisms.

The findings of this research advanced the scientific understanding of processes contributing to the degradation of chlorinated methanes under anoxic conditions and are relevant for bioremediation. The bacterial cultures derived, the microbiological activities reported, and the monitoring tools developed in this study will facilitate the design and successful implementation of bioremediation approaches at chlorinated methane-contaminated sites.